CLONING AND CHARACTERISATION OF A GENE ENCODING SEX-SPECIFIC TRANSCRIPTS IN *DROSOPHILA MELANOGASTER*

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THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

The University of Edinburgh

1996

DECLARATION

I declare that this thesis was composed by myself, and that the work described is my own, unless otherwise stated.

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July 1996

ABSTRACT

Sex determination of somatic tissue in *Drosophila melanogaster* is mediated by a hierarchy of regulatory genes controlled by the gene *Sex-lethal* (*Sxl*). Male differentiation is the default state, with *Sxl* being activated only in females. The Sxl protein is a RNA splicing modulator which regulates the splicing of both the *Sxl* transcript and the transcript of the sex-transforming gene *transformer* (*tra*). In the absence of Sxl protein, the *Sxl* and *tra* transcripts are spliced to produce transcripts incapable of producing active protein. Thus, active Tra protein is produced only in females, where it acts in concert with the product of the *transformer-2* (*tra-2*) gene to modulate splicing of the transcript from the *doublesex* (*dsx*) gene. In this way, sex-specific Dsx proteins are produced (Dsx^M and Dsx^F) which are differentially active, transcriptionally regulating sex differentiation genes, such as the *yolk protein* (*yp*) genes. Although the genes involved in sex determination have been well characterised, little is known about the genetic factors which play a part in sex-specific differentiation of the determined state. This thesis describes the cloning and characterisation of a gene which is a good candidate for a sex differentiation gene.

In an attempt to identify genes involved in differentiation of sex-specific characteristics, a non-gonadal differential screen was performed. Sex-specific, radiolabelled cDNA was used to screen a bacteriophage λ genomic DNA library and a genomic recombinant, fs(1), was selected for further analysis on the basis of preferential hybridisation to female non-gonadal cDNA. *in situ* hybridisation to *Drosophila* 3rd instar larva salivary gland polytene chromosomes indicates that the genomic DNA contained within fs(1) is located distally, on the left arm of chromosome 3, at position 061C1-3.

Two cDNAs, a 4.5kb cDNA (cDNA11) and a 3.0kb cDNA (cDNAa), were isolated on the basis of hybridisation to sequences contained within fs(1). Both cDNAs were fully sequenced and found to encode a novel OPA-repeat-containing

serine/threonine-specific protein kinase. cDNAa and cDNA11 both contain the entire open reading frame (ORF) which encodes this predicted protein, and differ only in untranslated regions. The cDNAa ORF was subcloned into a fusion-protein expression vector and fusion protein was successfully expressed in bacterial cells, as shown by Western blot analysis using antibodies specific to the vector-derived fusion protein. Genomic DNA, containing the entire cDNA11 and cDNAa transcription units, was isolated. The precise intron/exon structure of both cDNAs was determined by Southern blotting, DNA sequencing and PCR analysis.

cDNAa hybridises with four transcripts on Northern blots; a 3.0kb testis-specific, 3.5kb ovary-specific, 4.5kb female carcass-specific and a common transcript of around 4.7kb. The cDNA11-specific 3' UTR hybridises with the 4.5kb and common transcripts, but not with the 3.5kb or 3.0kb transcripts. Thus, cDNAa and cDNA11 are likely to represent the testis-specific and female carcass-specific transcripts, respectively. Both cDNAs contain translational control elements which are found in transcripts under male germline-specific translational control. The presence of these elements, together with Northern blot and whole-mount testis *in situ* hybridisation evidence, suggests that the testis-specific transcript is in fact germline-specific. Both cDNAs also contain *dsx*-like 13-nucleotide repeat elements which are required for Tra/Tra-2-mediated *dsx* splicing regulation. This, together with Northern blot evidence using *tra* and *tra-2* mutant flies, suggests that production of the female carcass-specific transcript is under direct control of *tra* and *tra-2*. Production of the testis-specific transcript is *tra/tra-2*-independent, as shown by Northern blots using *tra* and *tra-2* mutant flies and phenotypic analysis of these mutants.

The implications of these findings, and the possible functions of the protein encoded by cDNAa and cDNA11 are discussed.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Mary Bownes, for her help and guidance throughout the project. A heartfelt thank you to my fellow lab workers; Diane, Dot, Neil, Claudia, Angela (for her irrepressible happiness), Bryce (for his encyclopaedic knowledge), Debbie and Janis (for impromptu counselling), Elaine, Kathleen (for looking after my flies so well), Roger (for TVR discussions), Simone, Craig, Debiao and Wu-min. Thank you also, to all the ladies involved in media preparation and washing-up.

Above all, I want to thank my parents, Neil and Johanna, and my sister, Marie. Without their unfailing and unconditional support (both financial and emotional), I would never have made it through all the ups and downs of the last four years. This thesis is dedicated to them.

ABBREVIATIONS

amp	Ampicillin
ATP	Adenosine-5'-triphosphate
bp	Base pair
°C	Degrees centigrade
cDNA	Complementary deoxyribonucleic acid
Ci	Curies
cm	centimetre(s)
(d)dATP	2' (3'-di) Deoxyadenosine-5'-triphosphate
(d)dCTP	2' (3'-di) Deoxycytosine-5'-triphosphate
(d)dGTP	2' (3'-di) Deoxyguanosine-5'-triphosphate
(d)dTTP	2' (3'-di) Deoxythymidine-5'-triphosphate
dUTP	2' Deoxyuridine-5'-triphosphate
UTP	Uridine-5'-triphosphate
dNTP	Deoxynucleotide-5'-triphosphate
dH_2O	Distilled water
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Diaminoethanetetra-acetic acid
FSB	Formaldehyde sample buffer
g	gram
GST	Glutathione-S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

IPTG	Isopropyl-β-D-thiogalactoside
kDa	KiloDalton(s)
kb	Kilobase(s)
pfu	Plaque forming units
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends
pH	-Log10 (hydrogen ion concentration)
PolyA$^+$ RNA	Polyadenylated ribonucleic acid
PMSF	Phenylmethylsulphonyl fluoride
psi	Pounds per square inch
RNA	Ribonucleic acid
RNAase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
^{35}S	β-emitting isotope of sulphur
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	NNN'N'-tetra-methyl-1,2-diamino-ethane
Tris	Tris(hydroxymethyl)-amino-methane
Triton X-100	Octylphenoxypolyethoxyethanol
Tween-20	Polyoxyethylene sorbitan monolaurate
TCA	Trichloroacetic acid
U	Unit(s)
UV	Ultraviolet
υCi	Microcurie(s)

υg	Microgram(s)
υl	Microlitre(s)
υM	Micromolar
υmol	Micromole(s)
V	Volt(s)
v/v	Volume per volume
w/v	Weight per volume
Klenow	Large fragment of DNA ploymerase I
Krpm	Kilorevolutions per minute
λ	Lambda
L	litre
M	Molar
mA	Milliampere(s)
mCi	Millicuries
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
mmol	Millimole(s)
min	Minute(s)
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
MWt	Molecular weight
ng	Nanogram(s)
nmol	Nanomole(s)
OLB	Oligo labelling buffer

OD	Optical density
^{32}P	β-emitting isotope of phosphorus
pers. comm.	Personal communication
PEG	Polyethylene glycol
%	Percentage
pg	Picogram(s)
A	Alanine
R	Arginine
N	Asparagine
D	Aspartate
C	Cysteine
Q	Glutamine
E	Glutamate
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 SEXUAL DIMORPHISM IN *DROSOPHILA MELANOGASTER*

The difference between one cell type and another can be defined by two major criteria; the genes expressed by the cell and the morphology of the cell. This distinction is to some extent artificial, as the morphological development of the cell will be directed along specific pathways dependent upon the genes which are expressed in the cell. However, not all cells of the same morphology will have exactly the same patterns of gene expression. In the fruit fly, *Drosophila melanogaster*, the body tissues can be divided into groups dependent upon their morphology. Some of these tissues are present in both males and females and may appear to be identical. Some of these apparently identical tissues will, however, represent different cell types as defined by their patterns of gene expression, due to the fact that there are genes active in the tissue which define it as either male or female. Some tissues are present only in one sex. Thus we can divide the fly up into three major tissue types. These are sexually-dimorphic somatic tissues (present in both sexes), sex-limited somatic tissues (present in only one sex) and germline tissues.

There are essentially two components of the developmental process which lead to differences between these tissues. These are determination and differentiation. The available evidence shows us that somatic tissues are determined to be male or female via the action of a hierarchy of regulatory genes (reviewed in Baker 1989; Slee & Bownes, 1990; Burtis, 1993; Cline 1993, Ryner & Swain, 1995). The sex determination of the germline, while requiring some products of the same hierarchy is ultimately brought about by a set of different genes (reviewed in Steinmann-Zwicky, 1992).

Once a tissue or cell has been determined as male or female it must then use this information as a framework within which to interpret the various positionally and temporally restricted signals which tell any given cell or tissue where it is in the fly and what function it must perform. In this way, a specific pattern of gene expression

will be implemented which will eventually lead to the correct development of the tissue or cell. In order to understand how the sex determination decision is transduced into sex-specific characteristics, it is necessary to study the regulation and function of genes which are modulated by the sex determination decision. The isolation and characterisation of such sex differentiation genes is the subject of the work described in this thesis. It is first necessary to review the available evidence concerning the sex determination signal which is responsible for the development of sex-specific features. We will first consider the tissues of *Drosophila* which are morphologically or genetically sex-specific.

1.1.1 GROSS MORPHOLOGICAL DIMORPHISM.

In *Drosophila melanogaster* sexual dimorphism is manifest in a number of structures (figure 1.1). Females are larger than males and contain seven segments instead of six. The posterior segments are darkly pigmented in the male but not in the female. The male has a muscle in the fifth abdominal segment which is not present in the female (Muscle of Lawrence or MOL). Until recently this muscle was thought to be involved in curling of the abdomen during copulation. However, certain mutants of the *fruitless* gene lack this muscle but are still able to copulate (Taylor *et al.*, 1994). At present it is not clear exactly what role this muscle plays. The foreleg of the male exhibits a short row of thick bristles known as the sex comb. The genitalia and analia are also clearly distinguishable. The analia is derived from a single group of imaginal cells which will develop down either a male or female pathway, while the genitalia develop from two distinct populations of cells, with only one group developing in any given sex (Nöthiger *et al.*, 1977; Schüpbach *et al.*, 1978; Epper, 1981).

Although the fat bodies of males and females appears largely similar, the female fat body cells express the *yolk protein* (*yp*) genes which are required for oogenesis (reviewed in Bownes, 1994). Thus, this tissue is dimorphic at the genetic level.

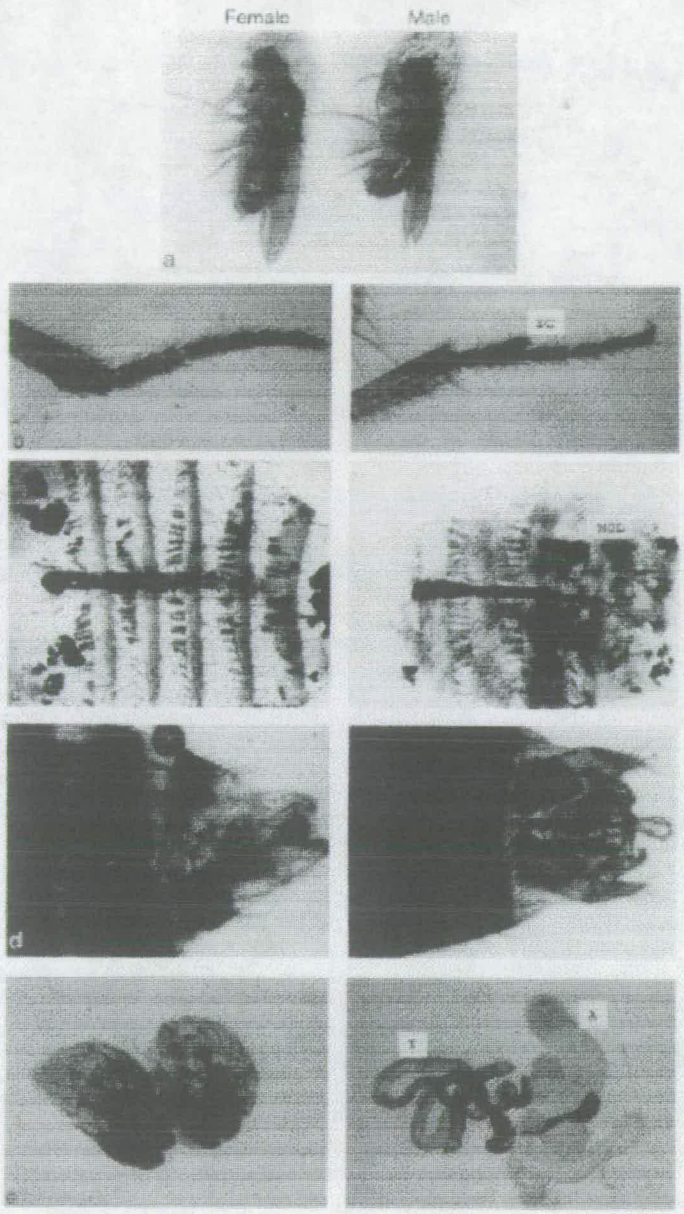


Figure 1.1
Gross morphological sexual dimorphism in *Drosophila* (taken from MacDougall *et al.*, 1995). (a) Males are smaller than females. (b) Males have a row of thickened bristles on the foreleg, the sex combs (SC). (c) There is a male-specific muscle in fifth abdominal segment, the muscle of Lawrence (MOL). (d) In males, the posterior abdomen is darkly pigmented. Genitalia and analia are also dimorphic. (e) Gonads are clearly dimorphic. A=Accessory gland, T=Testis.

1.1.2 NEUROLOGICAL DIMORPHISM.

The central nervous system of *Drosophila* exhibits several dimorphic characteristics. In the mushroom bodies of the brain, there are a greater number of cells in the female, including increased numbers of Kenyon fibres (Technau, 1984). Also, the terminal abdominal neuroblasts of the male undergo extra divisions which do not occur in the female (Taylor and Truman, 1992). It is not known what part these developmental differences play in determining the behavioural responses of the adult. However, it is interesting to note that perturbation of certain male courtship behaviours can result from feminisation of mushroom body tissue under the action of a transgene expressing the female from of a sex-determining gene (Ferveur *et al.*, 1995; O'Dell *et al.*, 1995). This will be discussed in more detail later in this chapter.

There are also sex-specific genital neurons which develop from the genital imaginal disc (Laugé, 1980). A dimorphic pattern of axon wiring and difference in the number of gustatory receptors is seen in the adult foreleg (Possidente & Murphey, 1989). It is also interesting to note that the development of the muscle of Lawrence depends not upon the sexual identity of the muscle itself, but of the innervating axons (Lawrence & Johnston, 1984; 1986). Thus, the axons which innervate this muscle must be sexually dimorphic, at least at the genetic level.

1.1.3 THE GONADS.

Gynandromorph studies indicate that the somatic component of the gonads develops from the same group of progenitor cells in both sexes (Szabad & Nöthiger, 1992). Clearly, the male and female gonads are highly structurally dimorphic, as would be expected (Figure 1.2). At the genetic level, there are also a large number of genes expressed sex-specifically in this tissue which are mainly involved in the differentiation of the germ cells into functional gametes. Gametogenesis has been well characterised in *Drosophila* and is discussed below.

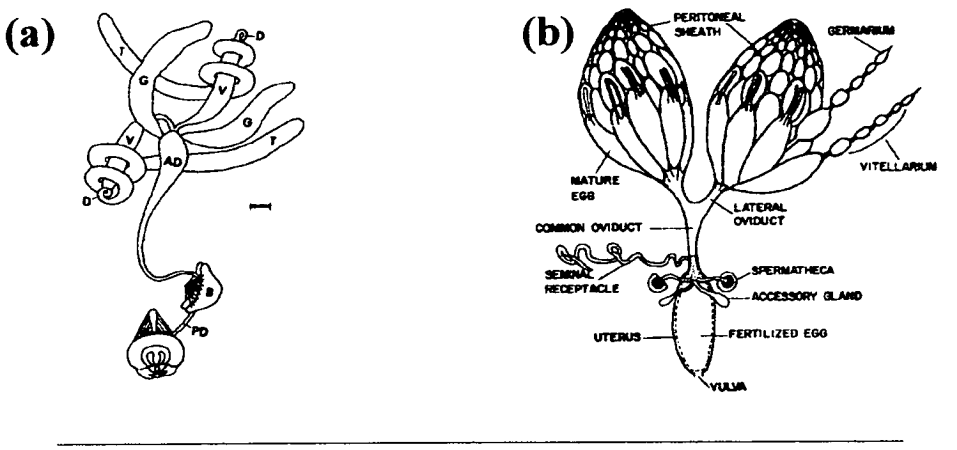


Figure 1.2
Structure of gonads in the male (a) and female (b) [taken from Mahowald & Kambysellis, 1980; Lindsley & Tokuyasu, 1980]. T=Testis, D=Testicular duct, V=Seminal vesicle, G=Accessory gland, AD=Anterior ejaculatory duct, B=Ejaculatory bulb, PD=Posterior ejaculatory duct. Scale in (a) represents 0.1mm.

1.1.3.1 Oogenesis.

The appearance of the *Drosophila* oocyte throughout oogenesis is shown in figure 1.3, and the major features of each stage are summarised in table 1.1 (Mahowald & Kambysellis, 1980).

Oogenesis begins in the germarium at the tip of the ovariole. A single oocyte progenitor cell, or oogonium, undergoes a mitotic division to produce two cells. The telophase of this division does not occur to completion, since cytokinesis is only partial and the cells remain joined by a cytoplasmic bridge. Each of the two cells undergoes another incomplete mitotic division, and so on until an interconnected 16-cell cyst is formed. Since cytoplasmic bridges only exist between mitotic 'partners', the 16-cell cyst contains only two cells which are linked by four cytoplasmic bridges. It is one of these two cells which will go on to complete meiosis and form the oocyte. The other 15 cells will develop as nurse cells, providing components to the oocyte which are required for successful oogenesis. One such nurse cell-supplied product is the mRNA from the gene *bicoid*

(Nüsslein-Volhard *et al.*, 1987). In a wild type oocyte, *bicoid* mRNA is supplied to the anterior of the oocyte by the nurse cells, where it is localised. The resulting gradient of Bicoid protein is responsible for determining the polarity of the embryo. In embryos mutant for the gene *dicephalic*, the oocyte is placed centrally in the follicle, with nurse cells at either end (Lohs-Schardin, 1982). This results in *bicoid* mRNA being supplied to both ends of the oocyte which causes the embryo to develop with bilateral symmetry, having a head at both ends.

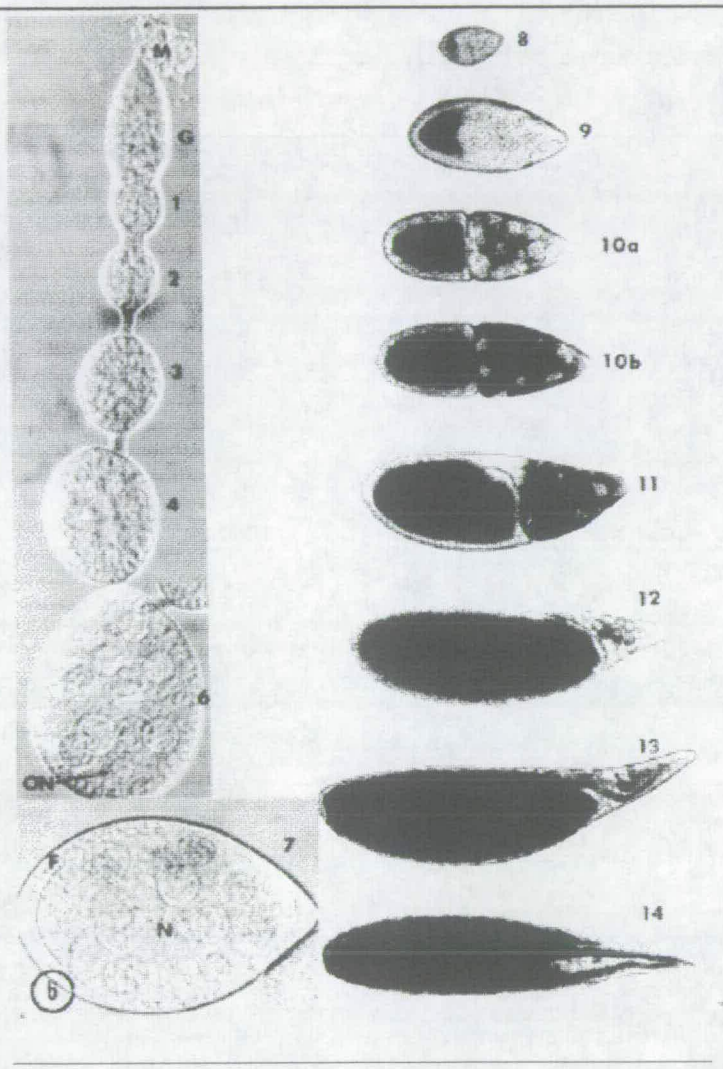


Figure 1.3
Light microscopy photographs of *Drosophila* oocytes throughout oogenesis (taken from Mahowald & Kambysellis, 1980). Stages 1-14 are indicated. Stages 1-7 @ X 380 magnification, using phase contrast microscopy. Stages 8-14 @ X 100 magnification, using bright field microscopy. M=Muscle sheath, G=Germarium, F=Follicle cells, N=Nurse cells, ON=Oocyte nucleus.

Stage	Duration (hours)	Size (υm)	Major Features
1	9.56		16-cell cyst in germarium.
2	9.56		Enters vitellarium. Nurse cell polyploidisation begins.
3	9.56		Oocyte nucleolus disappears.
4	9.16		Bulbous chromosomes in nurse cells.
5	2.61		Bulbous chromosomes disappear.
6	8.45		Cessation of follicle cell divisions
7	8.69	145	Elongation apparent. Follicle cells begin polyploidisation and become columnar over oocyte.
8	5.21	190	Yolk formation begins.
9	5.61	275	Most follicle cells over oocyte. Secretion of vitelline membrane begins. Border cells migrate.
10	5.13	430	Follicle cells are columnar over oocyte and squamous over nurse cells.
11	0.4	490	Nurse cells empty contents into oocyte. Chorion secretion begins.
12	1.9	540	Nurse cells shrunken. Formation of chorionic appendage begins.
13	1.79	650	Nurse cell nuclei begin to disappear.
14	1.98	700	Mature oocyte.

Table 1.1
Summary of the major features of stages 1-14 of oogenesis, as shown in figure 1.3.

At stage 1 of oogenesis, the follicle is present in the germarium and consists of the 16-cell cyst surrounded by somatic follicle cells. As the follicle develops into a mature oocyte, it passes down the ovariole until it is present in the oviduct. It is then fertilised by sperm stored in the spermatheca, and passes into the uterus to be laid as a fertilised egg.

At stage 2, the nurse cells begin to become polyploid, in preparation for the large amounts of maternal products which will be laid down in the developing oocyte. By stage 5 the follicle cells have divided 4 times and still completely cover the oocyte

and nurse cells. At stage 6, the follicle cells stop dividing and start to enlarge (stage 7) and become polyploid. Yolk starts to be deposited at stage 8 and by stage 9 the vitelline membrane begins to be laid down over the oocyte by the follicle cells. It is also at around stage 9 that the oocyte, which has been transcriptionally silent until then, begins some transcription and a cluster of cells migrate through the nurse cells and associate with the anterior of the oocyte to form the border cells. By stage 10 the follicle cells have enlarged and are squamous where they cover the nurse cells, but columnar over the oocyte. These columnar follicle cells begin to secrete the chorion at stage 11, and the nurse cells begin a rapid emptying of their contents into the oocyte. By stage 12, the now shrunken nurse cells form the 'anterior cap' of the oocyte, and the formation of the dorsal chorionic appendage begins. At stage 14, the chorion is completely formed and the oocyte is mature and ready for fertilisation.

1.1.3.1.1 The *chorion* Genes.

The chorionic membrane consists of around 20 proteins exported from the overlying follicle cells (Mahowald & Kambysellis, 1980). Genes have been cloned which encode six chorionic proteins; s36, s38, s15, s16, s18 and s19 (Spradling, 1981). The genes encoding s36 and s38 are X-linked, while the genes encoding the other four proteins are present in a cluster on chromosome 3. Within the follicle cells, these genes undergo 4-6 rounds of bi-directional replication which presumably is necessary to enable the production of sufficient chorion proteins. This replication event does not appear to be required for correct temporal regulation of *chorion* gene expression, but may act to regulate the levels of each chorion protein produced (Parks *et al.*, 1986).

1.1.3.1.2 The *yolk protein* genes.

The three yolk proteins YP1, YP2 and YP3 are synthesised in the female fat body and in the ovarian follicle cells (Bownes & Hames, 1978; Brennan *et al.*, 1980). The YP's synthesised in the fat body are carried to the ovary via the heamolymph, where

both the fat body-synthesised and follicle cell-synthesised YP's are taken into the oocyte via receptor mediated endocytosis (Bownes, 1986; Giorgi *et al.*, 1979).

Cloning of the genes encoding the YP proteins has revealed that all three genes are X-linked (Barnett *et al.*, 1980). The *yp1* and *yp2* genes are close together and divergently transcribed, while the *yp3* gene lies several kilobases downstream. The sex-specific regulation of these genes will be discussed later in this chapter.

Female flies which are starved after eclosion show greatly reduced levels of *yp* gene transcription (Bownes *et al.*, 1988). When these flies are fed, *yp* transcription levels return to normal. Transgenic studies delimited a 890bp fragment of DNA between *yp1* and *yp2* which is sufficient to confer this nutrient-dependent expression.

Expression of the *yp* genes is also regulated by the hormones juvenile hormone and ecdysone (Bownes *et al.*, 1993). Injection of 20-hydroxyecdysone into male flies results in transient expression of the *yp* genes in the fat body (Bownes *et al.*, 1983) and an analogue of the ecdysone receptor (ponasterone A receptor) binds to a DNA restriction fragment which lies between the *yp1* and *yp2* genes (Pongs, 1989, in Bownes, 1994). Fragments of DNA lying upstream, downstream and within *yp3*, and between *yp1* and *yp2*, have been isolated which confer 20-hydroxyecdysone-dependent induction on transgenic reporter gene constructs (Bownes *et al*, 1996).

Although ecdysone is able to stimulate *yp* expression in the fat body, a similar effect is not seen in the ovary follicle cells. A juvenile hormone analogue, however, is able to stimulate *yp* expression in both tissues (Jowett & Postlethwait, 1980). As yet, no specific DNA sequences have been identified which may be involved in the juvenile hormone response, and it is not known how this signal is transduced into regulation of the *yp* genes. Juvenile hormone analogue-dependent induction of transgenic reporter genes is only seen with *yp*-encoding constructs, suggesting that juvenile hormone may act at regulatory elements within the *yp* transcription unit, or may have

a role in post-transcriptional *yp* regulation (Bownes *et al*, 1996). It is not clear exactly what role hormone regulation has in the sex-specific patterns of *yp* gene expression seen in wild type flies, since natural levels of both juvenile hormone and ecdysone appear to be fairly constant in both sexes (Bownes, 1989).

1.1.3.1.3 The Maternal Effect Genes.

Both the *chorion* and *yp* genes are expressed maternally, with their protein products being supplied to the developing oocyte. Genetic analysis has revealed a large number of other 'maternal effect' genes, mutation of which results in oogenic/embryogenic defects. In particular, groups of maternal effect genes have been isolated which result in polarity defects such as ventralisation or dorsalisation in the developing embryo.

Cytoplasmic transplantation experiments indicate that there are factors localised at the anterior and posterior poles of the egg which established morphogen gradients, defining thoracic-head and abdominal structures respectively (Nüsslein-Volhard *et al.*, 1987). The genes responsible for these activities have been identified by screening for mutations which cause deletions of thoracic and abdominal regions. This analysis reveals genes which fall into three groups according to the areas of the embryo in which they specify polarity. These are the anterior, terminal and dorso-ventral maternal effect genes. Together, these genes lay down the anterior-posterior and dorsal-ventral polarity of the oocyte (and therefore the embryo), providing a framework of positional cues for the zygotically-expressed gap, pair-rule and segment polarity genes which determine the positional identity of embryonic cells within each developing segment.

The anterior polar centre is organised by the 'anterior group' of maternal effect genes. These are the genes *bicoid* (*bcd*), *exuperantia* (*exu*), *swallow* (*swa*), *bicaudal* (*bic*), *bicaudal-C* (*bic-C*) and *bicaudal-D* (*bic-D*) (St Johnston & Nüsslein-Volhard, 1992). *Bicoid* is a homeobox-containing protein which acts as a morphogen to define the

polarity of the anterior of the embryo (Berleth *et al.*, 1988). This is clearly shown by injection of *bicoid* RNA into ectopic sites of the embryo which results in head structures forming at the injection site (Driever *et al.*, 1990). *bicoid* transcript is localised to the anterior pole of the egg dependent on the gene products of the *exu*, *swa* and *staufen* genes (Berleth *et al.*, 1988; Stephenson *et al.*, 1988; St Johnston *et al.*, 1989). At fertilisation of the egg, translation of the RNA begins and gives rise to a gradient of Bicoid protein. That Bicoid is indeed a true morphogen is shown by the increase in anterior structures in response to increasing maternal *bcd* gene copies or injections of anterior cytoplasm (Struhl *et al.*, 1989). The mechanism by which these concentration-dependent decisions are made is shown by the presence of six *bcd* binding sites upstream of the *hunchback* gene (Driever & Nüsslein-Volhard, 1989). Three of these sites are of high affinity and three of low affinity. In this way the gene is activated in the anterior half of the embryo in response to *bcd* concentrations above a certain threshold level. Other possible targets of *bcd* include the gap genes *orthodenticle*, *empty spiracle* and *button head* which lead to anterior deletions when mutant (in St Johnston & Nüsslein-Volhard, 1992).

The 'posterior group' maternal effect genes direct the identity of the posterior region of the embryo and include the genes *nanos* (*nos*), *pumilio* (*pum*), *oskar* (*osk*) , *vasa* (*vas*), *tudor* (*tud*), *valois* (*val*) and *mago nashi* (St Johnston & Nüsslein-Volhard, 1992). Also required are the *staufen* (*stau*) gene and the zygotically-required genes *cappuccino* (*cap*) and *spire* (*spi*). *cap* and *spi* are involved in the localisation of the maternal products to the posterior pole of the egg (Manseau & Schüpbach, 1989; St Johnston *et al.*, 1991). This localisation takes place in a stepwise manner with each step dependant on the previous one. These maternal products go to make up part of the polar granules; densely staining structures which form at the posterior pole of the embryo and are incorporated into the pole cells at cellularisation. Maternal mutations which cause a lack of polar granules result in the production of sterile offspring due to a lack of germ cells. The mutant effect of all of the posterior group genes apart from *pum* and *nos* probably results from the inability to form these polar granules. Nanos protein is the likeliest candidate for the morphogen in this system, since in

nos mutants the polar granules are able to form but the posterior cytoplasm has no morphogen activity (Lehmann & Nüsslein-Volhard, 1991). Indeed, injection of *nanos* RNA into embryos from mothers mutant for all posterior group genes rescues the mutant phenotype (Wang & Lehmann, 1991). In *pum* mutants, polar granules form and rescuing activity is present. Thus, it seems likely that *pum* product is involved in the release of the *nos* morphogen (Lehmann & Nüsslein-Volhard, 1987). The only function of Nos appears to be the destablisation of maternal *hunchback* RNA which allows the activity of *hunchback*-repressed gap genes in the posterior half of the embryo (Irish *et al.*, 1989).

The 'terminal group' of maternal effect genes are responsible for the specification of the acron and telson (head and tail structures) of the embryo and their mode of action is very similar to that of the dorso-ventral genes which set up the dorso-ventral axis in the embryo. Both these sets of genes provide a spatially restricted ligand which is released from the follicle cells and recognised by a receptor which is present in the plasma membrane of the developing oocyte (see figure 1.4).

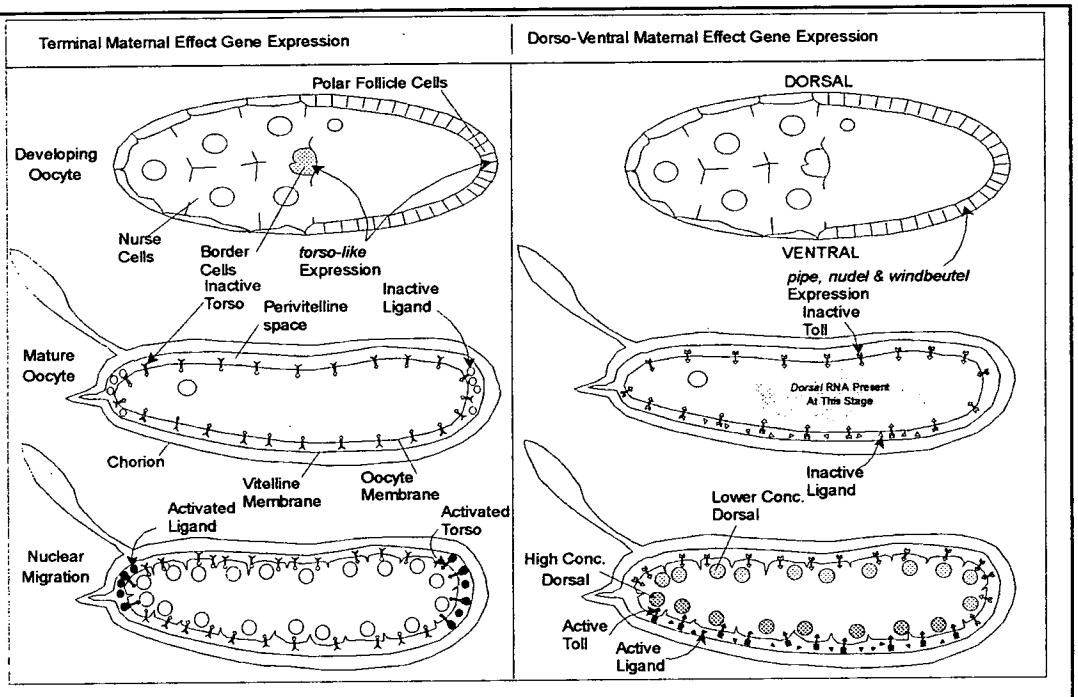


Figure 1.4
Diagram showing models for the maternal effect genes which are involved in setting up the terminal polarity and dorsal-ventral polarity of the *Drosophila* embryo. Major genes and proteins involved are indicated. See text for details.

The terminal group maternal effect genes are *torso* (*tor*), *torsolike* (*tsl*), *l(1)pole-hole* (*l(1)ph*), *fs(1)pole-hole* (*fs(1)ph*), *fs(1)Nasrat* (*fs(1)N*) and *trunk* (*trk*) (St Johnston & Nüsslein-Volhard, 1992). Cloning of the gene encoding Torso protein has revealed that it has homology to receptor tyrosine kinases (RTK's) and that the protein is localised all over the egg plasma membrane (Sprenger *et al.*, 1989; Casanova & Struhl, 1989). Constitutive gain-of-function alleles of this gene have shown a pattern of epistasis where the genes *trk*, *fs(1)N*, *fs(1)ph* and *tsl* lie upstream of *tor* and the gene *l(1)ph* lies downstream of *tor* (Ambrosio *et al.*, 1989; Stevens *et al.*, 1990). The somatic and germline specificity of the above mutants was shown by pole cell transplantations between embryos mutant for different genes. Thus *trk*, *fs(1)N* and *fs(1)ph* are required in the germline and *tsl* is required in the follicle cells but not the germline. Also, an X-ray induced *tsl/tsl* clone in the posterior polar follicle cells results in the loss of the posterior filzkorper structure. Thus, the current model suggests that *tsl* encodes the ligand for Tor and is released by the follicle cells at either pole of the embryo (figure 1.4). The polar identity of the follicle cells is believed to be defined by processes involving the genes *notch* (*N*) and *Delta* (*Dl*), since hypomorphic alleles of these genes can result in mislocalisation of *bicoid* to the posterior of the embryo as well as the anterior (Ruohola *et al.*, 1991). *trk*, *fs(1)N* and *fs(1)ph* which are released from the oocyte, may be required for the activation of Tsl which then binds to and activates Torso. Torso protein may then act to phosphorylate L(1)ph which in turn would go on to direct transcription/regulation of various downstream genes such as the terminally-expressed *tailless* and *huckebein* gap genes (Weigel *et al.*, 1990).

The 'dorso-ventral group' maternal effect genes act to determine the dorsal-ventral polarity of the embryo and include the genes *gastrulation-defective*, *snake*, *easter*, *spatzle*, *toll*, *tube*, *pelle*, *dorsal*, *cactus*, *pipe*, *nudel* and *windbeutel* (St Johnston & Nüsslein-Volhard, 1992). The genes *gastrulation-defective*, *snake*, *easter*, *spatzle*, *toll*, *tube*, *pelle* and *dorsal* belong to a group of genes which cause a dorsalising of the embryo when hypomorphic and a ventralising effect when hypermorphic. Mutant alleles of the genes *cactus*, *pipe*, *nudel* and *windbeutel* ventralise the embryo when

hypomorphic or amorphic and dorsalise it when hypermorphic. *toll* encodes a transmembrane receptor with homology to the interleukin-1 (Il-1) receptor and is present ubiquitously in the oocyte plasma membrane (Hashimoto *et al.*, 1988; 1991). Again, constitutive gain-of-function alleles of *toll* have enabled the epistatic relationship between these genes to be elucidated (Anderson *et al.*, 1985). The somatically-required genes *pipe*, *nudel* and *windbeutel* together with the germline-required genes *gastrulation-defective*, *snake*, *easter* and *spatzle* lie upstream of *toll* while the germline-required genes *tube*, *pelle*, *cactus* and *dorsal* lie downstream of *toll*. Injection of perivitelline fluid from *toll/toll* flies induces the embryo to develop with its ventral side at the site of injection, if the mother is mutant for *pipe*, *nudel* or *windbeutel* (Stein *et al.*, 1991). *toll/toll* mothers must be used to provide this fluid as otherwise, the inducing ligand is sequestered from the fluid by the Toll receptor. This is in agreement with a role for these genes in the production of ligand from ventral follicle cells and explains their mutant phenotypes.

The nature of the Toll ligand is not known, but injection of perivitelline fluid can rescue the mutant phenotypes of embryos from *snake*, *easter* and *spätzle* mutant mothers (Stein & Nüsslein-Volhard, 1992, in St Johnston & Nüsslein-Volhard, 1992), suggesting that these three genes may encode the ligand or have a role in ligand production. The observation that the *snake* and *easter* genes encode putative serine proteases suggests a possible role for these gene products in activation of the ligand by proteolytic cleavage (DeLotto & Spierer, 1986; Chasan & Anderson, 1989). The end result of the functions of all of these gene products is the setting up of a nuclear gradient of Dorsal protein such that ventral nuclei contain higher concentrations of Dorsal than dorsal nuclei (Roth *et al.*, 1989). The use of *cactus/dorsal* double mutants to produce intermediate nuclear concentrations of dorsal has indicated that the downstream targets of dorsal are *zerknült* (*zen*) and *decapentaplegic* (*dpp*) which are active at low Dorsal concentrations and *twist* and *snail*, which are active at high Dorsal concentrations (Roth *et al.*, 1989). This also shows that Dorsal is a true morphogen, producing different concentration-dependent effects. The homology of Cactus to Iκβ protein and the homology of Dorsal to

NF-κβ suggests a model for Cactus, Dorsal and Toll protein action based on the function of the homologous proteins as shown in figure 1.5 (St Johnston & Nüsslein-Volhard, 1992). This model has been shown to be essentially correct (Norris & Manley, 1996) and will be discussed further in chapter 5.

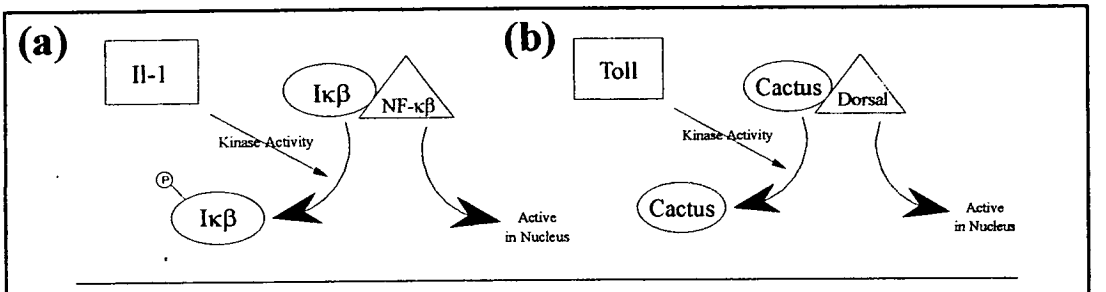


Figure 1.5
Diagram showing mode of action of Il-1, Iκβ and NF-κβ, (a), and a proposed model for Toll, Cactus and Dorsal proteins, (b), based on the shared homology of these proteins. Other factors may lie between Toll and Cactus- no direct phosphorylation of Cactus is indicated.

1.1.3.2 Spermatogenesis.

In oogenesis, each meiosis gives rise to only one haploid oocyte, with the other two products of the meiotic divisions becoming polar bodies which eventually degenerate. However, in the testes, four functional haploid spermatozoa are produced from each meiosis.

1.1.3.2.1 Generation of Haploid Spermatids.

Spermatogenesis begins at the very tip of the testis (see figure 1.2) with a small number of diploid stem cells. These stem cells undergo four mitoses before they enter meiosis and spermatogenesis (reviewed by Lindsley & Tokuyasu, 1980). The first mitotic stem cell division gives rise to a second stem cell, which remains at the tip of the testis, and a primary spermatogonial cell, which completes the further 3 mitoses and eventually gives rise to 64 spermatozoa. The 64 progeny cells from each primary spermatogonial cell develop synchronously, moving down the testis towards the testicular duct as they develop. The primary spermatogonial cell is surrounded by

two somatic cyst cells which remain with the germ cells throughout their divisions. The initial mitotic divisions of the primary spermatogonial cell produce a cyst containing 16 primary spermatocytes. These cells remain connected by intercellular bridges (ring canals), in the same way as the 16 cells produced from the initial stem cell divisions of oogenesis. The diploid primary spermatocytes undergo a 90 hour growth phase during which their volume increases approximately 25 times. This growth phase is followed by two meiotic divisions, producing 64 haploid spermatids which are still linked by ring canals.

1.1.3.2.2 Spermiogenesis.

The process by which a spermatid differentiates into a spermatozoon is called spermiogenesis, which lasts 5-6 days and comprises 3 main phases known as elongation, individualisation and coiling (reviewed by Lindsley & Tokuyasu, 1980).

Prior to the main phase of elongation a pre-elongation phase occurs. During pre-elongation the mitochondria of the spermatid assemble into a bipartite laminate sphere known as the nebenkern. During the second meiotic division, the centriole does not replicate, leaving a single centriole present in the spermatid. This centriole forms the 'basal body', at the base of the nucleus. The axoneme begins to extend from the basal body and is surrounded by plasma membrane to form a cilium. The acrosome begins to be formed from Golgi body which repositions at the anterior pole of the nucleus.

During elongation the spermatid cyst extends to the point where it occupies 80-90% of the length of the testis. The axoneme greatly extends and becomes associated with cylindrical rods formed from the nebenkern. The nucleus metamorphoses from a sphere into a needle-like structure.

During individualisation, the ring canals linking the spermatids are broken down. A swelling, known as the cystic bulge, moves down the length of the sperm bundle, sweeping away the ring canals as well as any excess organelles and cytoplasm.

Coiling is the final process in spermiogenesis. One of the two cyst cells becomes associated with the membrane at the base of the testis and the sperm bundle moves down the testis as it is coiled. The coiled spermatozoa then separate from the cyst cells and move to the seminal vesicle.

1.1.3.2.3 Genetic Factors Involved in Sperm Production.

Much less is known about the genetic control of sperm formation than about oogenesis. However, it has been known for some time that the Y chromosome is required for correct spermatogenesis, since XO males develop normally in all respects other than in spermatogenesis, where severe meiotic disruption is observed (Lifschytz & Hareven, 1977). Such males are sterile. Six Y-linked complementation groups (known as fertility factors) are responsible for the role of the Y chromosome in spermatogenesis (Kennison, 1981; Hazelrigg *et al.*, 1982; Gatti & Pimpinelli, 1983). No functional sperm are produced in males lacking either the whole Y chromosome or one or more of the fertility factors; *ks-1*, *ks-2*, *kl-1*, *kl-2*, *kl-3*and *kl-5*. Elongation does occur, but spermatids degenerate before reaching maturity. The three fertility factors *ks-1*, *kl-3* and *kl-5* correspond to thread-like structures seen in spermatocytes which are thought to represent large Y chromosome loops at these loci which may have some structural role in spermatogenesis (Bonaccorsi *et al.*, 1988).

Approximately 20% of EMS-induced X-linked male sterile mutants exhibit some level of meiotic disruption in spermatogenesis. In a characterisation study of male-sterile mutants, Lifschytz & Hareven, 1977, described 3 categories of spermatogenic defect which result in male sterility; namely, timing mutations (nebenkern formation occurs before, rather than after, meiosis), spindle structure

mutations and primary spermatocyte deformation mutations. Of the first group, the *ms(1)413*, *ms(1)682* and *ms(1)RD11* X-linked lesions cause the nebenkern to form prior to meiosis and result in a perturbation of its structure. Nebenkern stability also appears to be affected, since disaggregation is seen during elongation. The *ms(1)516* X-linked lesion characterises the second group. This mutation causes a perturbation of the spindles seen during the second meiotic division where only one set of centrioles is seen, rather than two. Of the third group, the *ms(1)401* X-linked lesion appears to cause primary sprematocytes to become stalled prior to meiosis. An accumulation of primary spermatocyte late stages is seen, although some elongation does take place. In addition, the stalled spermatocytes show structural abnormalities such as fragmentation of nucleoli.

In a screen performed by Schäfer, 1986a, five genes with male gonad-specific transcripts were isolated. Only one of these transcripts, encoded by the *Mst87F* gene (originally *mst(3)gl-9*), was not detected in germline-deficient males, indicating that this transcript is germline-specific. The other four transcripts were shown to be localised to accessory gland tissue. The diploid primary spermatocytes are highly transcriptionally active during their growth phase (Olivieri & Olivieri, 1965). After this stage however, virtually no transcription is seen to occur. Clearly, all of the mRNA required to make protein during spermiogenesis of the haploid spermatids must be stored in the cells prior to meiosis. We might expect translational control of these transcripts to be required to coordinate the expression of spermiogenic proteins. Translation from the *Mst87F* transcript has been shown to be under translational control during spermiogenesis (Kuhn *et al.*, 1988). Although the *Mst87F* transcript is present in the primary spermatocyte, Mst87F protein does not appear until late in spermatid elongation. Transgenic studies have shown that a 24bp section of the *Mst87F* 5' UTR is sufficient to confer this translational control upon a reporter gene (Schäfer *et al.*, 1990). This translational control element (TCE) is the subject of further discussion in chapter 4. The *Mst87F* gene belongs to a family of genes known as the *Mst(3)CGP* genes (Schäfer *et al.*, 1993). They all encode proteins with cysteine/glycine/proline repeats, all contain a TCE element at an

invariant position and are all translationally regulated. The *Mst(3)CGP* genes encode structural sperm tail proteins which may form part of the axoneme (Kuhn *et al.*, 1988).

The UTR sequences of the anterior group maternal effect gene *exuperantia* (*exu*) are also implicated in transcript regulation in the male germline (Hazelrigg & Tu, 1994). The *exu* gene function is required in the male germline for correct spermatogenesis since *exu* mutant males are sterile, exhibiting disfunctional non-motile sperm (Hazelrigg *et al.*, 1990). Both testis-specific and ovary-specific *exu* transcripts contain the same coding potential, but differ at their 5' and 3' ends, with the testis-specific transcript having an extended 3' UTR (Hazelrigg & Tu, 1994). The *exu* allele, exu^{DP3}, results in production of a testis-specific *exu* transcript which lacks most of the testis transcript-specific 3' UTR and causes male sterility but has no effect on oogenesis (Hazelrigg & Tu, 1994). Deleting specific regions of the *exu* testis transcript-specific 3' UTR results in male sterility and reduced levels of *exu* mRNA (Crowley & Hazelrigg, 1995). Thus, the function of the extended 3' UTR may be to stabilise the *exu* transcript in a testis-specific manner.

The sex determining gene *tra-2* is also required in the male germline for correct spermatogenesis, as mutant males are sterile, producing non-functional sperm (Belote & Baker, 1983). However, this effect is at least partially indirect, since *tra-2* has been shown to be required for efficient production of the male testis-specific *exu* transcript (Hazelrigg & Tu, 1994).

Some progress has been made in elucidating the transcriptional regulation of primary spermatocyte-expressed genes by the identification of specific regulatory elements in the *β2 tubulin* gene promoter (Michiels *et al.*, 1989). The β2 tubulin protein is the major tubulin isotype which forms part of the primary spermatocyte cytoskeleton and is expressed only in the testis. Transgenic analysis shows that 53bp of *β2 tubulin* gene upstream sequence together with the first 23bp of the transcription unit is sufficient to confer primary spermatocyte-specific expression upon a reporter gene.

Further deletion of the 53bp upstream sequence shows that a 14bp section which has been called the β2 upstream element 1 (β2UE1) is absolutely required for this transcriptional regulation. Identification of other genes containing the β2UE1 in their promoters will hopefully enable further elucidation of the genetic processes required for primary spermatocyte development and spermiogenesis.

We would expect that a process such as spermatogenesis would be highly dependent upon precise control of the cell division cycle and this is indeed the case. A simplified model showing the major processes involved in cell cycle regulation is shown in figure 1.6. Sigrist *et al.*, 1995, have shown that a separate CDC25 phosphatase, the Twine/CDC25 phosphatase, is required in male germ cells to drive the cells into M-phase. It is possible that this protein is required in male germ cells to compensate for the lack of the String/CDC25 phosphatase which is maternally supplied to female germ cells. Flies mutant for *twine* or *cdc2* (encoding the p34 kinase) still go through spermiogenesis even though the chromosome segregation and cytokinesis of meiosis is incomplete (White-Cooper *et al.*, 1993; Sigrist *et al.*, 1995). This indicates that cell-cycle progression is to some extent independent of spermiogenesis. However, four genes have been identified, in which mutations cause the primary spermatocytes of mutant male flies to stall in growth phase, which is in fact an extended G2-phase (Lin *et al.*, 1996). These genes are *spermatocyte arrest* (*sa*), *cannonball* (*can*), *always early* (*aly*) and *meiosis 1 arrest* (*mia*). None of these genes are required for female fertility. The authors suggest that the process of primary spermatocyte entry into meiosis and subsequent spermiogenesis can be broken down into two separate pathways; those which are *twine*-dependent (such as meiotic chromosome condensation and cytokinesis) and those which are *twine*-independent (entry into spermiogenesis). The persistence of Cyclin A protein in *sa*, *can*, *aly* or *mia* mutant spermatocytes suggests that the products of these genes may be involved in the ubiquitin-mediated pathway which usually degrades Cyclin A in a cell cycle-regulated manner.

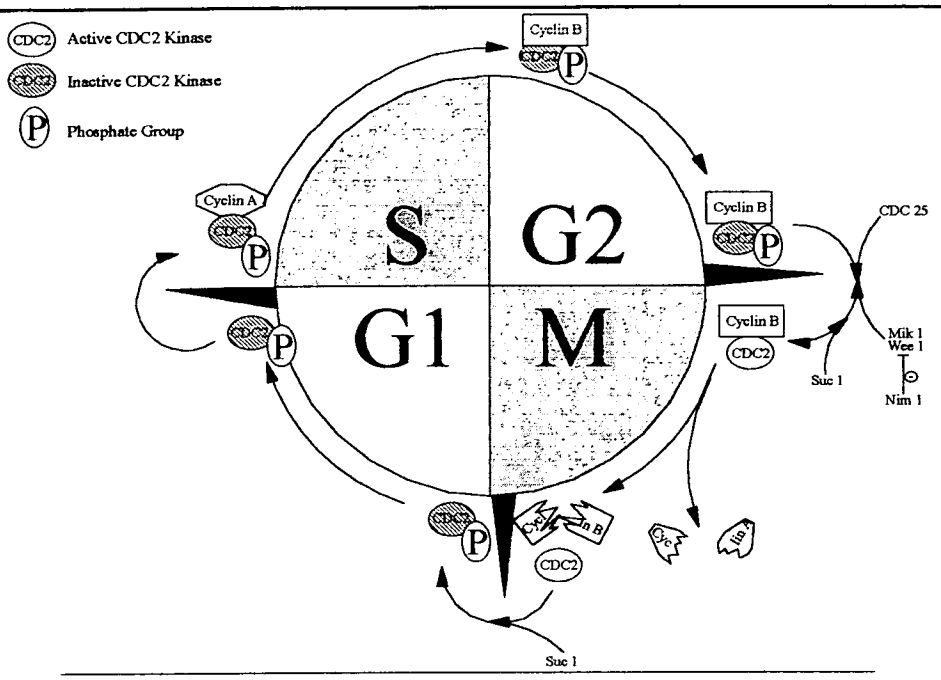


Figure 1.6
Diagram showing the major gene products involved in cell division cycle regulation (reviewed in Pines & Hunter, 1990; Woodgett, 1991; Enoch & Nurse, 1991; MacNeill *et al.*, 1991; Glover, 1991). A great deal of this work was carried out on the fission yeast *S. pombe*, but homologous genes have been found in a number of organisms, including humans, and have been shown to be functionally interchangeable in a number of cases. S=S-phase (DNA synthesis phases), M=M-phase (Mitotic division phase), G1=Gap-phase 1, G2=Gap-phase 2.

The CDC2/p34 Serine/Threonine (S/T) kinase is thought to be the main 'workhorse' of cell cycle regulation, acting to drive the cell through the divisions between the phases of the mitotic cycle, dependent upon its phosphorylation state and association with the cell cycle-regulated Cyclin proteins. Dephosphorylation of CDC2 in the CDC2/CyclinB complex in G2-phase enables transition from G2 into M-phase. Dephosphorylation of CDC2 is brought about by the CDC2-activator, CDC25 phosphatase. The Wee1 protein (and its redundant homologue Mik1) is a Serine/Threonine/Tyrosine (S/T/Y) kinase which inhibits cell cycle progression and may phosphorylate CDC2. The Nim1 S/T kinase is thought to negatively regulate Wee1 via phosphorylation. Suc1 protein physically associates with CDC2 and regulates G2/M transition (negatively) and mitotic progression (positively).

It is clear that *Drosophila* exhibits a large number of sexually dimorphic characteristics. In the following section the role of genetic factors in determining the sex of the developing fly is discussed.

1.2 DETERMINATION OF THE SEX OF SOMATIC TISSUE.

The origin of the sex determination signal was discovered by C.B.Bridges, 1921, via studies of the progeny of matings between triploid females and diploid males. During oogenesis in triploid flies one haploid set of chromosomes is distributed to the oocyte pronucleus and one haploid set to each of the three polar bodies. This still leaves one haploid set of chromosomes unaccounted for. The remaining chromosomes are randomly apportioned to either the oocyte or the polar bodies. When such flies are mated to normal males progeny can be generated with an unusual number of X chromosomes relative to numbers of sets of autosomes (the X:A ratio). This work revealed that the sex of flies is dependent upon the X:A ratio, such that flies with a ratio of 4X:4A, 3X:3A, 2X:2A or 1X:1A (an X:A ratio=1) are female while flies with a ratio 1X:2A (an X:A ratio=0.5) are male. It was also observed that flies with an X:A ratio lying between these two values such as 2X:3A (an X:A ratio=0.67) developed neither as complete males nor complete females but as intersexes, consisting of mosaic patches of male and female tissue (Bridges, 1925). The Y chromosome was seen to play no part in somatic sex determination but it is required for the development of fully functional sperm.

The mechanism by which the X:A ratio is assessed has been very well characterised. Several genes which are involved in the transduction of the X:A ratio have been identified on the basis of their mutant phenotypes as shown in table 1.2 (reviewed in Baker & Belote, 1983; Slee & Bownes, 1990). This genetic analysis revealed two major types of sexual transformation. Firstly, the conversion of females to pseudomales. Pseudomales exhibit male characteristics displaying male pigmentation and cuticular structures and possessing rudimentary testes. They are, however, of female size and infertile due to non-functional sperm. Triploid (2X:3A) intersexes appear to represent a decision made early on in development by each cell to follow either the male or female pathway of development. This is indicated by the variability of patch size in these mosaics with a large patch representing a decision made early on in development and thereafter followed autonomously by the progeny

of that cell. Conversely, *dsx* mutants are true intersexes with the phenotype seeming to result from an attempt at the individual cell level to express both male and female functions. Thus, true intersexes are non-mosaic flies with each cell being neither male or female but of an intermediate phenotype.

GENE	MUTATION	PHENOTYPE
Sex-lethal	Recessive	Female lethal. XX cells masculinised
	Dominant	Male lethal. XO cells femininised
transformer	Recessive	Females transformed to pseudomales
transformer-2	Recessive	Females transformed to pseudomales
doublesex	Recessive	Females transformed to intersexes
	Recessive	Males transformed to intersexes
	Recessive	Both sexes transformed to intersexes
	Dominant	Females transformed to intersexes
intersex	Recessive	Females transformed to intersexes

Table 1.2
Summary of phenotypes caused by alleles of the sex-determining genes *Sex-lethal*, *transformer*, *transformer-2*, *doublesex* and *intersex*.

Evidence from a number of studies has shown that the genes discussed above operate in a hierarchy to determine sex. The epistatic relationship between these genes was first shown by mating flies which were homozygous for either *dsx* or *tra* mutant alleles, resulting in progeny homozygous mutant for both genes (Baker & Ridge, 1980). These *tra/tra*, *dsx/dsx* flies exhibited a phenotype typical of *dsx/dsx* mutants, indicating that *tra* and *dsx* gene products act in the same pathway and that *dsx* is epistatic to *tra*. In another set of experiments, *tra/tra* flies which were also mutant for other genes in the hierarchy were crossed to a strain containing a P-element construct with the hsp70 constitutive promoter fused to the female-specific *tra*

coding sequence (McKeown, *et al.*, 1988). Ectopic *tra* expression was shown to cause development down the female pathway in *tra/tra* flies lacking *Sxl* function but the phenotypes of *tra-2* and *dsx* mutations were unaffected. This shows that *tra* is epistatic to *Sxl* and that *tra-2* and *dsx* are epistatic to *tra*. In addition, the transforming activity of this *tra* construct was shown to be dependent upon the integrity of the *ix* gene, which shows that *ix* product is required for the correct progression of female differentiation. A pattern of epistasis was established which suggested that these genes act in a hierarchical pathway in the following way; *Sxl–>tra–>tra-2–>dsx*. Since the function of the *dsx* gene is dependent upon the *ix* gene, *ix* may lie downstream of *dsx*, or may act in conjunction with *dsx* (Baker & Ridge, 1980).

The epistatic relationship described above is based mainly on the genetic evidence, but is confirmed by the molecular evidence that active *Sxl* is required for production of relevant *tra* transcripts and that *tra* and *tra-2* are required for production of relevant *dsx* transcripts (Nagoshi *et al.*, 1988). However, this evidence does not rule out the possibility that *tra-2* actually lies on a side-branch of the hierarchy, acting in conjunction with *tra* and upstream of *dsx*. Cloning and molecular characterisation has elucidated the mechanistic relationship between *Sxl*, *tra*, *tra-2* and *dsx*, and is discussed below.

1.2.1 DOSAGE COMPENSATION AND *SEX-LETHAL*

In *Drosophila melanogaster* the fact that males have one X chromosome while females have two, is compensated for by a change in the transcription rate of the X-linked genes (Mukherjee & Beermann, 1965). Incorporation of tritiated uridine into hnRNA being transcribed from polytene chromosomes indicates that the single male X chromosome is hypertranscribed.

The lethality of mutations at the X-linked *Sxl* locus indicates that this gene must serve functions other than sex determination. Such lethality cannot be entirely

explained by incomplete development due to transformation of sex in such mutants, since *tra/tra* pseudomales still require active *Sxl* product to be viable. It has been observed that in XX larvae carrying hypomorphic alleles of *Sxl*, the rate of gene expression of the X chromosome is increased (Lucchesi & Skripsky, 1981). It was also observed that heterozygosity for a *Sxl* hypomorph can partially rescue the mutant phenotype of a hypomorphic allele of the X-linked segmentation gene *runt* in XX larvae, indicating that a reduction in *Sxl* product leads to an increase in *runt* gene product (Gergen, 1987). This evidence points towards a female-specific role for *Sxl* in deactivation of X-chromosome hypertranscription.

If the above model is correct then it follows that *Sxl* should be inactive in this respect in males and this is shown to be the case by the following evidence. Females homozygous for a *Sxl* hypomorph die as embryos but this mutation has no effect on males (Cline, 1984). Indeed, males carrying a deletion covering the *Sxl* locus develop normally. However, males with a hypermorphic *Sxl* allele are embryonic lethal while females homozygous for this allele are unaffected. The lethality of a *Sxl* hypomorph in the female must be due to the presence of two X chromosomes rather than the absence of the Y as the allele is also lethal in XXY flies.

Further evidence for a role for *Sxl* in dosage compensation as well as sex determination is seen in *X/X, Sxl/Sxl* X-ray induced clones of cells which, as well as being male-like in character, are also much smaller than male-like clones caused by other transforming mutations (Sänchez & Nöthiger, 1982). This reduced viability in *Sxl/Sxl* clones is proposed to be due to over expression of the X chromosomes. The same inviability is seen in the XO cell clones of gynandromorphs heterozygous for a hypermorphic *Sxl* allele (Cline, 1979). In such flies, the XO clones which carry the *Sxl* mutation are much smaller than those that do not. Thus, it would appear that *Sxl* is active only in females to turn off X chromosome hypertranscription.

Four other genes have been proposed to be the targets of *Sxl* in the dosage compensation pathway. These are the *male-specific lethal* genes *1*, *2* and *3* (*msl-1*, *2*,

and *3*) and the gene *maleless* (*mle*) which kill only males when homozygous mutant (Belote & Lucchesi, 1980a; 1980b; Lucchesi *et al.*, 1982). These genes' involvement in the dosage compensation pathway and their epistasis to *Sxl* is indicated by the partial rescue of the phenotype of *X/X, Sxl/Sxl* flies by mutant alleles of *msl-1*, *2*, *3* and *mle* (Skripsky & Lucchesi, 1982). Since the mutant phenotypes of *msl-1*, *msl-2* and *mle* mutant flies are similar to the phenotype observed when all three are mutant at the same time, it is likely that these genes all act in the same pathway (Bachiller & Sánchez, 1989; Baker *et al.*, 1994). The *mle* gene bears homology to RNA and DNA helicases, supporting a role for this protein in increase of transcription rate as part of the transcription complex (Kuroda *et al.*, 1991).

Dosage compensation of the male X is a global process, affecting the whole chromosome, as shown by rearrangements which bring genes from elsewhere in the genome under the dosage compensation control of this chromosome and also the loss of dosage compensation of genes translocated from the X chromosome to autosomes (Lucchesi & Manning, 1987; Sass & Meselson, 1991, in Henikoff & Meneely, 1993). Immunofluorescence using anti-*mle* antibody showed that Mle protein is associated with hundreds of sites along the male X chromosome but not the female X chromosome (Kuroda *et al.*, 1991). This same association is also seen with Msl-1 and Msl-3 proteins and is associated with an increase in acetylated histone H_4 (Baker *et al.*, 1994). This is in agreement with a direct role for these proteins in hypertranscription. Further support for this comes from the observation that active Sxl protein prevents Mle protein binding to the female X and that *msl-1* , *msl-2* and *msl-3* are all required for this binding to take place in the male (Gorman *et al.*, 1993).

It has also been proposed that Mle may act to up regulate the transcription of other groups of autosomal genes in a non sex-specific manner. This is based on evidence which identifies a temperature sensitive allele, nap^{ts}, as an allele of *mle* (Kernan *et al.*, 1991). At the restrictive temperature, nap^{ts} causes paralysis in male and female flies. It is thought to do this by causing a down regulation of transcription from the

para locus which encodes a sodium channel protein believed to be involved in the creation of action potentials. This function of *mle* is genetically separate from its function in dosage compensation and may represent a redundant function which is primarily carried out by other homologous genes, but the localisation of Mle protein to autosomal polytene arms indicates that there may be an alternative function for Mle in regulating the expression of certain autosomal loci (Kuroda *et al.*, 1991; Kernan *et al.*, 1991; Gorman *et al.*, 1993).

Based on this evidence it seems likely that a function of active *Sxl* in the female is to inactivate X chromosome transcriptional hyperactivation by inactivating the pathway which includes the products of the *msl-1*, *msl-2*, *msl-3* and *mle* genes. Analysis of *msl-2* transcripts has shown that, in females, active Sxl protein acts to prevent the removal of a leader sequence in the *msl-2* transcript which may prevent active Msl-2 protein from being produced, since Msl-2 is only produced in male flies (Zhou *et al.*, 1995). In contrast, the transcripts from the *msl-1*, *msl-3* and *mle* genes are not sex-specific (Palmer *et al.*, 1994; Baker *et al.*,1994). Therefore, *msl-2* is the target of *Sxl* in its dosage compensation function.

1.2.2 *SEX-LETHAL* DETERMINES SEX.

Clones homozygous for a *Sxl* null allele can be introduced into flies by use of X-ray induced mitotic recombination during embryogenesis (Sánchez & Nöthiger, 1982). As shown in table 1.2, the cells comprising these clones become masculinised despite their XX genotype. It was also observed that XO clones in gynandromorphic flies can be transformed from male-like to female-like by the presence of a hypermorphic *Sxl* Allele (Cline, 1979). Taken together with the fact that male flies show no requirement for *Sxl*, these results show that active *Sxl* product is responsible for the determination of cells to follow the female pathway of differentiation and that in males the absence of active *Sxl* directs the embryo down the male (default) pathway. Since the X:A ratio is the primary signal which determines sex and since

Sxl lies at the head of the hierarchy of sex determining genes, it seems likely that transduction of the X:A ratio occurs via the activation of the *Sxl* gene.

1.2.2.1 Activation of *Sex-Lethal* : Zygotic and Maternal Factors.

Sxl activation was originally proposed to require the presence of X-linked 'numerator' elements. These would exert either an activatory or repressive effect upon *Sxl*. The presence of an equal number of X chromosomes to sets of autosomes would then modulate this effect to permit *Sxl* activation. This could occur in a number of ways. Initially it was thought possible that these elements were non-coding and acted as binding sites for autosomally-encoded repressors (or 'denominators') which were 'watered down' by the extra X-linked sites provided in a fly with an X:A ratio=1 (Chandra, 1985). However, it is also feasible that the numerator elements encode proteins which act in some way to suppress the negative effects of autosomally encoded repressors. Also, a role for activation of *Sxl* by virtue of an extra copy of *Sxl* together with co-operative effects and possible autoregulation of the numerators would be feasible.

Two X:A numerators were identified on the basis of duplications and deletions which are sex-specific lethal (Cline, 1986; 1988). These are the sisterless loci, *sis-a* and *sis-b*. The *sis-a* gene encodes a protein with homology to the basic leucine zipper (bZip) family of transcription factors (Erickson and Cline, 1993). Initially, *sis-a* was uncovered as a recessive allele which was selectively lethal to females and caused an increase in the amount of male tissue present in 2X:3A mosaic intersexes. It was also found that a duplication of this locus was lethal to males but only if they carried two wild-type copies of *Sxl*. The *sis-b* gene encodes a protein containing a helix-loop-helix amphipathic dimerisation domain and a basic DNA-binding region (basic HLH domain, or bHLH) (Cline, 1988; Torres & Sánchez, 1989). This locus was isolated as a duplication which was also lethal to *Sxl/Sxl* males and resulted in an increase in the female character of 2X:3A intersexes. It was observed that the two loci had a synergistic relationship and that deletion of one copy of each of these loci

resulted in female lethality. These double heterozygote flies exhibit sterility due to ovarian tumours. This phenotype is also seen in germline clones homozygous for a *Sxl* null allele (Schüpbach, 1985). The above observations indicate that these elements act in a dosage dependant manner to activate the *Sxl* gene. Another numerator element, *sis-c*, has also been identified but has much weaker effects than either *sis-a* or *sis-b* (Cline, 1993).

Another locus was found to have a role in *Sxl* activation, being required both maternally and zygotically (Steinmann-Zwicky, 1988). This is the *liz* locus (previously named both *fs(1)1621* and *sans-fille*). In gynandromorphic clones of XO cells carrying a *liz* duplication, an increase in the female character of the cells was observed (Steinmann-Zwicky & Nöthiger, 1985). The opposite was seen in such clones carrying a deletion covering this locus. The effect of deleting one copy of *liz* in female flies heterozygous for a *Sxl* null allele is to cause a degree of masculinisation and also to decrease the viability of the flies by an amount dependent upon the maternal genotype at this locus. This indicates that maternal *liz* is required for activation of the dosage compensation function of *Sxl*. Zygotic *liz*, however, would appear to have its role in the activation of the sex-determining function of *Sxl*. The protein encoded by *liz* bears extensive homology to certain human SnRNP proteins and so is likely to be involved in the positive regulation of *Sxl*-mediated RNA processing (Flickinger & Salz, 1994). The proposal that *Sxl* is epistatic to these elements is further supported by two pieces of evidence. Firstly, the masculinisation and lethality of females double heterozygote for both *liz* and *Sxl* null alleles is rescued by a constitutive *Sxl* allele and secondly, the male lethality of a *sis-a* and *sis-b* duplication is rescued by a *Sxl* null allele (Steinmann-Zwicky, 1988; Cline, 1988).

In addition to the zygotic requirement for numerator elements, there is also a maternal requirement for *Sxl* activation. As well as maternal *liz*, two genes have been identified as temperature sensitive mutations that selectively kill only the daughters of female flies carrying the allele. These have been named *daughterless* (*da*) and

Daughter-killer (*Dk*) (Bell, 1954; Steinmann-Zwicky *et al.*, unpub. in Slee & Bownes, 1990).

Daughters from *Dk* mothers do reach adulthood if they are of the genotype 2X:3A, presumably due to the alleviation of dosage compensation problems that this genotype permits. These daughters do, however, consist of much more male-like tissue than is normally the case, indicating a reduction in the efficiency of the female sex determination pathway. That this reflects a loss of *Sxl* activation is shown by the fact that lethality is rescued by a *Sxl* constitutive gain-of-function allele. However, *Dk* may well act via other genes, since temperature shift experiments show that the requirement for *Dk* product ends before the end of oogenesis when *Sxl* is still dependent upon positive regulation from other genes. The effects of *Dk* are pleiotropic as it can also cause lethality in males (Steinmann-Zwicky *et al.*, unpub. in Slee & Bownes, 1990).

Triploid (2X:3A) embryos from *da/da* mothers have much more male tissue than is normally seen in intersexes of this type (Cline, 1983). It appears that this is caused by a reduction in efficiency of *Sxl* activation, as demonstrated by the following evidence. Firstly, daughters from *da/da* mothers have unusual *Sxl* gene expression patterns, exhibiting a greatly increased concentration of male-specific *Sxl* transcripts (Maine *et al.*, 1986). In addition, temperature shifts using a temperature-sensitive *da* allele have shown that *da* product is required from the mother until the end of the blastoderm stage when *Sxl* is thought to commence autoregulation (Gergen, 1987). Perhaps the most conclusive evidence for a role for *da* in *Sxl* activation comes from the observation that daughters of *da/da* mothers no longer die if the daughters carry a *Sxl* constitutive gain-of-function allele (Cline, 1983). Also, the male lethality caused by a *sis-a* and *sis-b* duplication, which can be rescued by a null *Sxl* allele, can also be rescued by a maternal *da/da* phenotype (Cline, 1988). Thus, *Sxl* is epistatic to *da* and *Dk* which are required for *Sxl* activation in females.

The *da* gene encodes a number of overlapping transcripts with apparently redundant functions (Cronmiller *et al.*, 1988). Sequence analysis of cloned cDNA's has revealed that the predicted *da* protein bears homology to proteins of the helix-loop-helix (HLH) class, notably *myc*, *MyoD1* and the *T3*, *T4* and *T5 achete-scute* complex gene products (Caudy *et al.*, 1988; Murre *et al.*, 1989). These proteins contain a HLH amphipathic helix dimerisation domain and in some cases a basic DNA binding domain. The Da protein is of the bHLH type. The *myc* gene product has been implicated in both transcriptional regulation and RNA processing.

The bHLH proteins are believed to bind to DNA as dimers where they act to modulate gene expression. The potential for a great deal of regulatory variety is inherent in this type of protein as it may function as a dominant negative by having a non-functional or inappropriate DNA binding domain or it may serve several different functions by interacting with other proteins to form heterodimers which then go on to modulate gene expression in different ways. In this respect, it is interesting to note that zygotic *da* is also a proneural gene where it acts to determine between epidermal and neuronal cell fates by positive and negative interactions with the HLH proteins encoded by the genes *extramacrochaetae* (*emc*), *hairy* (*h*) and the genes of the *achete-scute* complex. Also, it has been determined that the *sis-b* numerator element is, in fact, the *T4* locus of the *achete-scute* complex (Erickson & Cline, 1991). In early neurogenesis, the *T4* gene product is acted upon negatively by both the *h* and *emc* gene products, and the *achete scute* and *da* gene products positively regulate each other (Vaessin *et al.*, 1990). This clearly lends weight to the model of the numerator elements as encoding positive regulators of *Sxl* which act together with maternal *da* protein to overcome repressive activities encoded by autosomal denominators. It has been shown that Sis-b protein forms complexes with Da protein in the *Drosophila* embryo (Deshpande *et al.*, 1995). The same study showed that Sis-b protein is expressed prior to *Sxl* activation and that Sis-b enters that nucleus just before *Sxl* transcription begins. In addition, by use of a hsp70/*sis-b* fusion it was shown that *sis-b* is lethal to males due to expression of Sxl in these flies (Torres & Sánchez, 1991). Neither *T3* nor *T5 achete-scute* loci appear to

function as strong numerators, as shown by anomalous expression of these loci. Indeed *T4* appears to be the major transcript of the complex, since *T3* and *T5* are only poorly expressed by comparison and only activate *Sxl* very weakly (Parkhurst *et al.*, 1993).

If *hairy* is ectopically misexpressed at around the same time as *Sxl* initiation, female lethality results (Parkhurst *et al.*, 1990). The observation that this lethality can be rescued by duplications of the *sis-a* or *sis-b* elements points towards the possibility that autosomally encoded HLH proteins may act to antagonise the *sis-b* function in *Sxl* activation, since this is the type of protein-protein interaction in which Hairy is involved during neurogenesis. The pan neural gene *deadpan (dpn)* has been proposed as such a denominator (Younger-Shepherd *et al.*, 1992). *deadpan* encodes a bHLH protein which is dependent upon zygotic *da* product for its expression in neuroblasts. It was seen that an inappropriately low dosage of *dpn* loci relative to *sis-b* loci caused male lethality while the opposite imbalance caused female lethality. The non-HLH protein encoded maternally by the *groucho* gene interacts with Dpn and these two proteins may act together as *Sxl* repressors (Paroush *et al.*, 1994). It was also seen that male viability was reduced by maternal mutations of *emc*, another HLH protein-encoding gene which is expressed at around the blastoderm stage. The male lethalities discussed above were rescued by a *Sxl* null allele, showing that *dpn* and *emc* act to repress *Sxl*. This suggests that maternal *emc* in the male performs the converse function to maternal *da* in the female, acting to repress *Sxl*.

The model for *Sxl* activation which presents itself is one whereby positively acting maternal and numerator-encoded HLH proteins interact with repressive autosomal denominator-encoded HLH proteins to assess the X:A ratio and either activate or repress *Sxl*. However, the observation that the segmentation gene *runt* also acts as a numerator shows that not all proteins involved in this process are of the HLH class (Duffy & Gergen, 1991). The mode of action of *runt* is different from that of the other numerators. Female embryos which are mutant for *runt* still express *Sxl* at the termini of the embryo. It has been proposed that Runt may act indirectly upon *Sxl* to

regulate its expression in a spatially restricted manner. However, the mode of action of the other two numerators, *sis-a* and *sis-b* is most likely to be the transcriptional activation of *Sxl*.

The zinc finger-containing protein encoded by the *hermaphrodite* (*her*) locus also appears to be required maternally for *Sxl* activation (Pultz *et al.*, 1994; Pultz & Baker, 1995; Ryner & Swain, 1995). The *her* locus was identified as a recessive mutation which caused reduced viability and reciprocal transformations in both sexes, with the severity of both of these effects dependent upon the maternal *her* genotype (reviewed in Baker & Belote, 1983). Zygotic *her* mutations have no effect upon *dsx* expression and cannot be rescued by ectopic expression of either *Sxl* or *tra*. This suggests that the zygotic function of *her* lies either downstream of, or parallel to *dsx*.

1.2.2.2 Activation of *Sex-Lethal* is a Two Part Process.

In order to understand the mechanism of *Sxl* activation, it is helpful to first appreciate that it is a two-step process yielding two slightly different sets of transcripts-the early and late/adult transcripts (Bell *et al.*, 1988). The first set appears early on in the embryo at around nuclear division 8 and disappears after cellularisation. *Sxl* alleles which carry a late transcript-specific deletion show adult *Sxl* function defects but can be rescued by a *Sxl* allele which specifically lacks early *Sxl* functions (Maine *et al.*, 1986; Cline, 1986). This suggests that the early *Sxl* transcripts may direct dosage compensation but that the late transcripts are required for the sex determining function of *Sxl*. The transcripts which lead to sex determination appear before the disappearance of the early transcripts and are present throughout the life of the fly (Samuels *et al.*, 1991). Maps of the exons included in these transcripts are shown in figure 1.7.

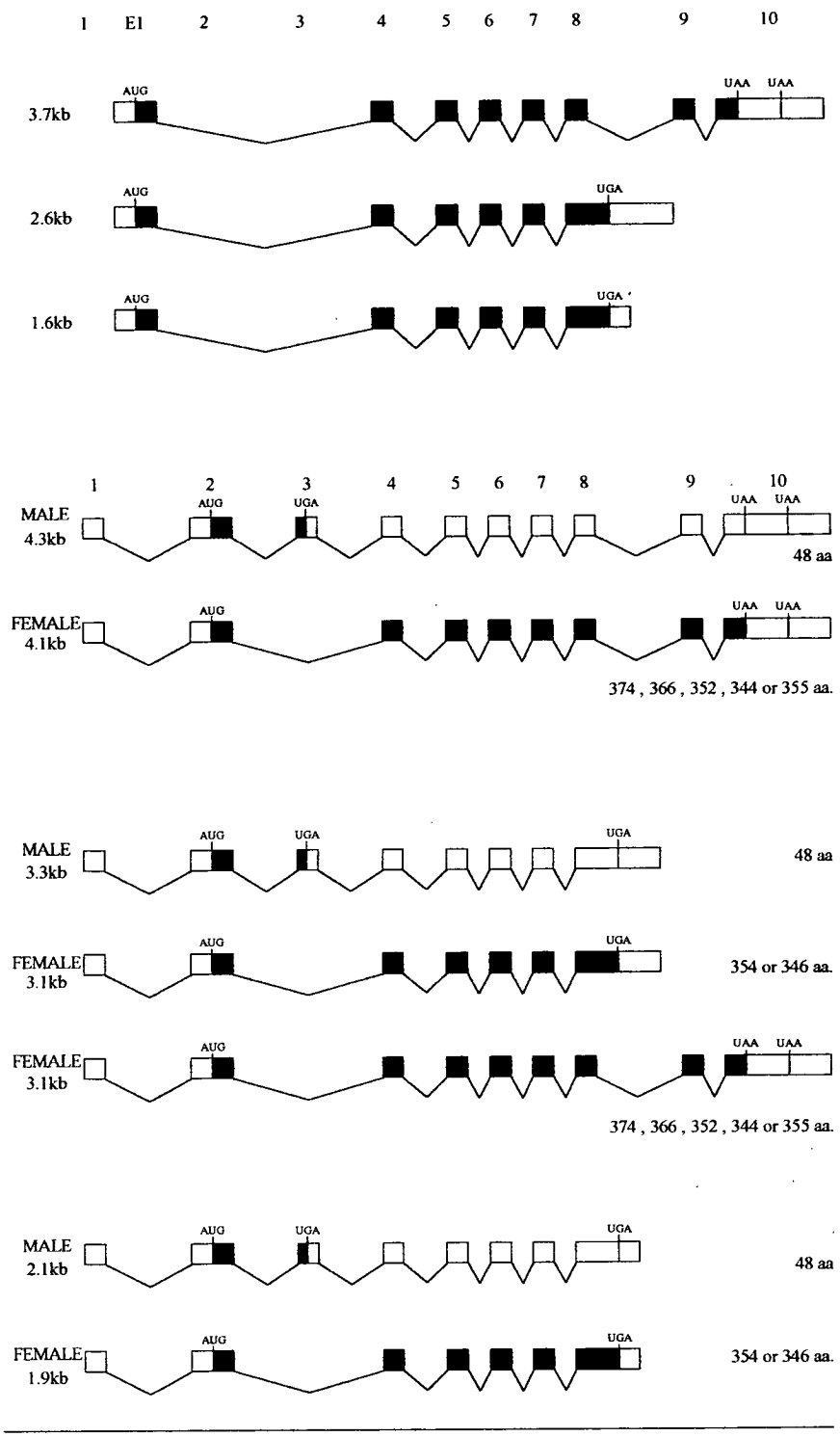


Figure 1.7

Exon/Intron structure of *Sxl* early and late transcripts. Exon numbers are shown. Sizes of spliced transcripts are indicated in kilobases. Open reading frames are shown as shaded areas and the number of amino acids encoded by each reading frame is also shown. Transcripts which are sex-specific are indicated.

The first part in the process to activate *Sxl* is a result of the reading of the X:A ratio by the numerator/denominator elements and maternally-encoded factors such as Da and Dk. That Da is a factor in the production of the early *Sxl* transcripts is shown by low levels of these transcripts in *XX* embryos from *da/da* mothers (Maine *et al.*, 1986). These transcripts are entirely female-specific. As can be seen in figure 1.7, the early transcripts are initiated from a separate promoter (P_E) which is downstream of the late promoter (P_L) from which the late transcripts are initiated. Lac-Z reporter gene fusions driven by P_E provide stage-specific expression of β-galactosidase in wild type embryos but not in embryos from *da/da* mothers (Keyes *et al.*, 1992). Similar reporter gene studies have shown that the products of the *da*, *sis-a*, *sis-b* and *runt* genes all activate transcription from P_E, while the product of the *deadpan* gene is a P_E-repressor (Estes *et al.*, 1995). The P_E promoter contains 19 repeats of the CANNTG consensus known to act as a binding site for bHLH proteins. A cluster of six of these repeats at the proximal end of P_E is essential for reporter gene expression in the assay described above (Estes *et al.*, 1995).

At around cellularisation, transcription form the P_L promoter begins and this is maintained throughout development and into adulthood (Maine *et al.*, 1986). It is not known what is responsible for expression of the late transcripts but it is not dependent upon the sex determination hierarchy as it is also transcribed in the male slightly after transcription begins in the female. What is under the control of the hierarchy is the sex-specific splicing of exon 3 from the late transcripts (Bell *et al.*, 1988). This exon contains translational stop codons and is not spliced out of the mRNA in the male which results in the production of a non-functional truncated protein. In the female, the splicing out of this exon allows production of a large, functional Sxl protein containing two domains which bear homology to RNA binding proteins (Bell *et al.*, 1988). It has been observed that temperature sensitive *Sxl* alleles which lack the 5' end of the gene (including the late promoter) can complement another *Sxl* allele which lacks sex determining function and usually only produces male-specific late transcripts (Keyes *et al.*, 1992). This indicates that

the production of the sex determining transcripts (i.e. the female-specific late transcripts) depends upon the early transcripts produced initially in the embryo.

The factor responsible for the splicing out of *Sxl* exon 3 in the female is in fact Sxl protein itself (Sakamoto *et al.*, 1992). *Drosophila* cell culture cotransfection experiments using constructs producing active Sxl protein and minigene constructs containing *Sxl* genomic DNA between exons 2 and 4 have shown that Sxl protein is responsible for the exclusion of exon 3 in the processed RNA molecule. Various Uridine sequences have been identified in the vicinity of exon 3 (figure 1.8).

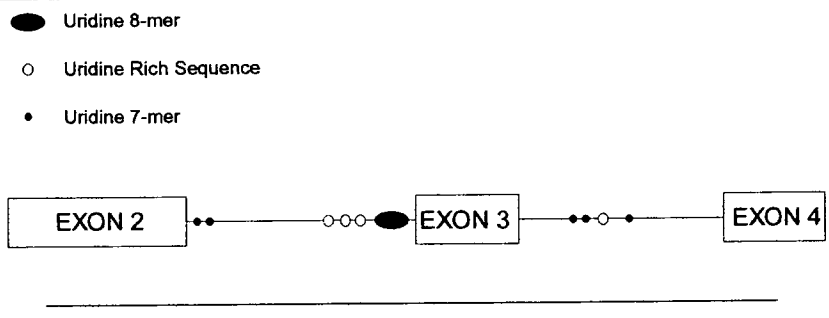


Figure 1.8
Diagram showing approximate locations of uridine-rich sequences around the sex-specific splice site of the *Sxl* transcript.

It has been observed that deletion of several of these U-rich motifs prevents the splicing function of *Sxl* and this function can be restored by replacing the deleted sequences with synthetic uridine oligonucleotides (Sakamoto *et al.*, 1992). Sxl protein has been purified (Samuels *et al.*, 1994). This protein has been used for gel-shifts, UV crosslinking and footprinting experiments which directly demonstrate that Sxl binds to polyU runs in RNA. Wang and Bell, 1994, have shown that binding of Sxl to polyU runs is a cooperative process, mediated by the amino-terminus of the protein.

Germline transformation with *Sxl* minigenes has been used to study the precise sequences which are required for Sxl-mediated splicing of exon 3 (Horabin &

Schedl, 1993a; 1993b). Somewhat surprisingly, deletion of the polyU runs downstream of exon 3 disrupted exon3-splicing much more than deletion of the upstream runs. In addition, when the donor splice site at the 3' end of exon 3 is deleted, exon 3 is not removed. This suggests that the major site of Sxl-mediated 'blocking' is at the donor splice site of the intron between exons 3 and 4. However, a model which involves the sequestration of the whole region between exons 2 and 4, mediated by cooperative Sxl binding to polyU runs in the introns, cannot be ruled out at this stage.

The above system enables us to explain the observations previously made with respect to the timing and cell autonomous nature of the sex determination decision, as described below.

Sex of the fly is not determined until cellularisation. This is shown by the fact that in gynandromorphs, XO clones develop correctly with correct dosage compensation (i.e. hypertranscribed) and sex (i.e. male), only if the second X chromosome is lost *before* cellularisation (Sánchez & Nöthiger, 1983). XO clones which are generated by loss of the X chromosome *after* cellularisation exhibit dosage compensation and sexual characteristics characteristic of XX cells, which causes these clones to be small and of low viability. However, these clones can be rescued by a *Sxl* null allele, indicating that the source of the autonomy lies with the *Sxl* gene (Sánchez & Nöthiger, 1983). Perhaps the most definitive demonstration of this comes from XX flies which have been manipulated such that *Sxl* is less likely to be activated, where patches of tissue develop which are either male or female in nature but never a mixture of the two (Cline, 1985).

The autonomy of the sexual choice at the cellular blastoderm stage is demonstrated by several phenomena. A number of structures such as the male foreleg sex combs have been examined in triploid intersexes and it has been determined that a decision is made at the level of the individual cell to produce either the male or female version of the structure and that this decision is then carried through to completion

(Hannah-alava & Stern, 1957). The variation in clone size seen in triploid intersexes clearly reflects the precise stage at which the progenitor cell of the clone becomes determined, in the same way as the earliness of the loss of the ring X in a gynandromorph is in proportion to the size of the resulting XO clone (Cline, 1984).

The above phenomena can be explained as follows. In females, the X:A ratio is transduced by the numerator, denominator and maternal loci, as discussed above, such that the proportions of these gene products, together with the double dose of the *Sxl* gene, allows initiation of transcription from the *Sxl* P_E promoter. It is highly likely that the bHLH Da and Sis-b proteins, together with the Sis-a, Sis-c and Runt, act directly to overcome the repressive effect of Deadpan and Groucho and enhance expression from this promoter. Initiation from this promoter prevents the inclusion of exon 3 which allows the production of active Sxl protein. Thus, when transcription from the late promoter commences, Sxl protein produced from the early transcripts prevents the inclusion of exon 3 into *Sxl* late transcripts. In this way, an autoregulatory loop is set up that ensures that Sxl protein is continually produced and inherited by progeny cells. Thus, the sex determination decision is set to the female pathway in each of these progeny cells. In the male, active Sxl protein is not produced, due to the fact that the initial transcription of *Sxl* from P_E is not activated due to the X:A ratio not permitting a sufficient concentration of the relevant activator proteins with respect to copies of the *Sxl* locus. A summary of this model for *Sxl* sex-specific splicing is shown in figure 1.9(a) and 1.9(b) (upper panels).

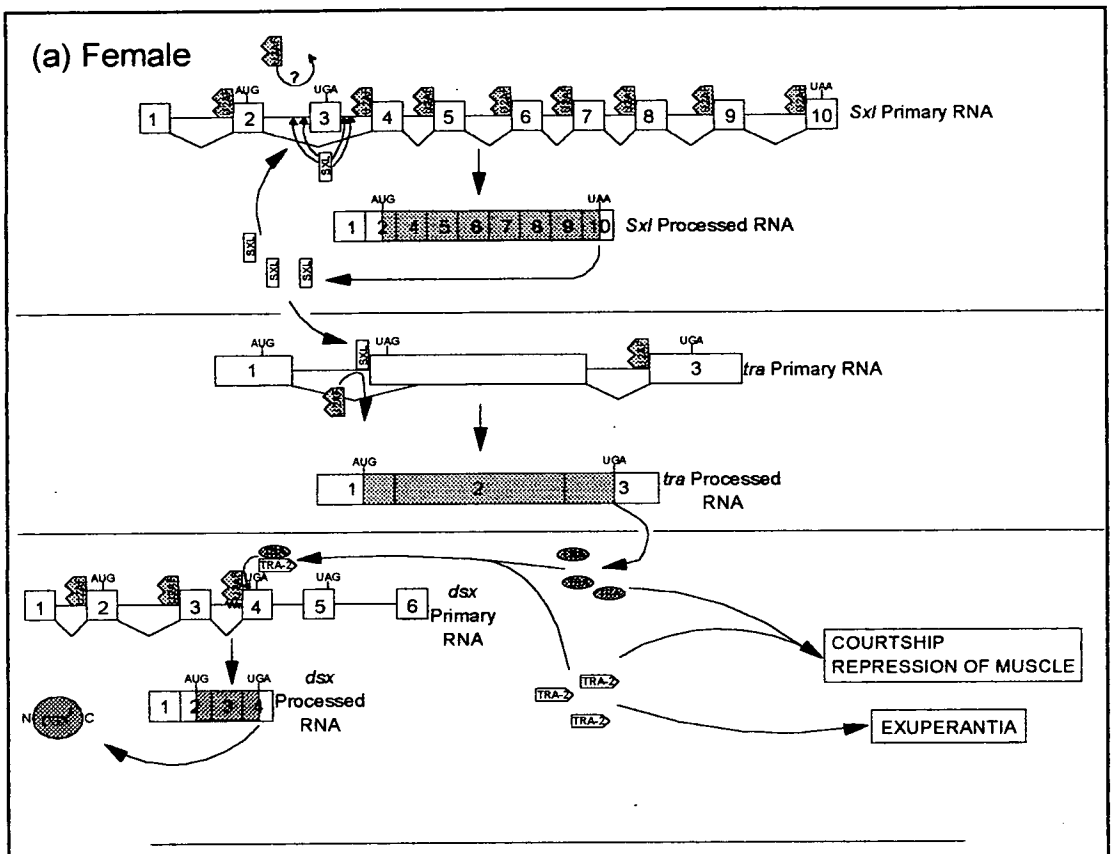


Figure 1.9 (a)

Sex-specific alternate splicing of *Sxl*, *tra* and *dsx* transcripts in the female (taken from MacDougall *et al.*, 1995). Exons are indicated by boxes with open reading frames shaded. Proteins involved in the splicing regulation and produced by it, are shown. Of the splicing apparatus, only the U2AF splicing factor is shown. DSX^F=Dsx female-specific protein, DSX^M=Dsx male-specific protein, SXL=active Sxl protein, TRA=active Tra protein, TRA-2=Tra-2 protein.

In the female, Sxl prevents inclusion of exon 3 in the *Sxl* mRNA, allowing more active Sxl protein to be produced. Sxl also blocks the acceptor splice site at the 5' end of exon 2 in the *tra* transcript, causing splicing which produces a *tra* mRNA encoding a full-length active Tra protein. Tra and Tra-2 promote usage of the *dsx* exon 4 acceptor site, resulting in a female-specific *dsx* transcript.

(Figure 1.9 (b) overleaf.)

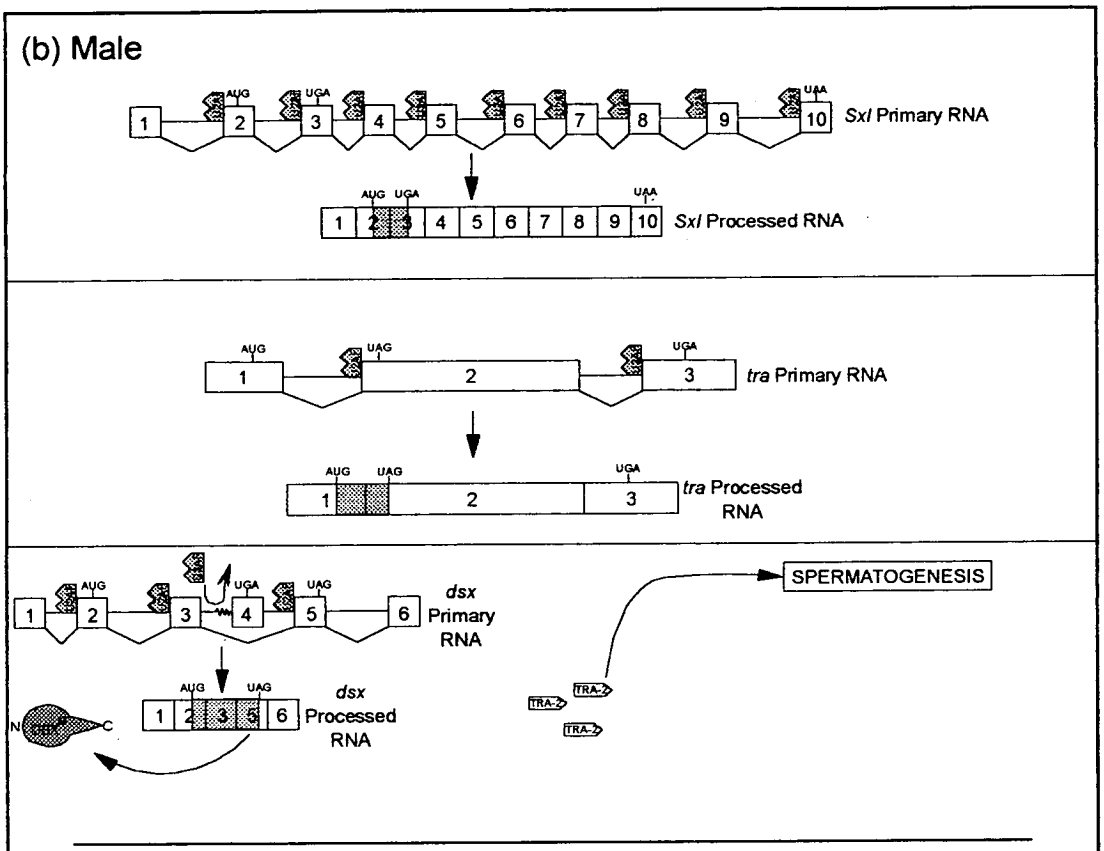


Figure 1.9 (b)

Sex-specific alternate splicing of *Sxl*, *tra* and *dsx* transcripts in the male (taken from MacDougall *et al.*, 1995). Exons are indicated by boxes with open reading frames shaded. Proteins involved in the splicing regulation and produced by it, are shown. Of the splicing apparatus, only the U2AF splicing factor is shown. DSX^F=Dsx female-specific protein, DSX^M=Dsx male-specific protein, SXL=active Sxl protein, TRA=active Tra protein, TRA-2=Tra-2 protein.

In males, lack of early Sxl protein enables default splicing which results in *Sxl* and *tra* mRNAs which encode non-functional, truncated proteins and a male-specific Dsx protein.

The cell autonomy of the sex determination decision is analogous to that seen in position effect variegation (PEV) in *Drosophila*. In PEV, chromosomal rearrangements bring certain genes close to terminal or centric heterochromatin. This results is a mosaic expression pattern reflecting a cell-autonomous decision to either express or not express the gene (Tartof *et al.*, 1989; Henikoff, 1990). In the case of the *white* gene, this results in the eyes of the fly having a mottled appearance caused by the mosaic expression of the gene in this tissue. Perhaps the most likely model for this effect is that the ON/OFF decision is affected by a flowing of heterochromatin across the locus in some cells but not in others. The state of the gene would then be 'locked' at some point in development via the action of certain non-histone chromosomal proteins (NHC's). Evidence for this includes the reduction of PEV clone by the addition of heterochromatic material (i.e. extra copies of the Y chromosome) to the cell, presumably due to a sequestering of NHC's by this material (Dimitri & Pisano, 1989). Also several suppressor mutations of PEV have been isolated and have been identified as encoding proteins important in the formation and maintenance of heterochromatin, such as the *suvar(2)5* gene which encodes a protein containing the 37aa 'chromodomain' which has been implicated in the binding of proteins such as Heterochromatin associated protein-1 (HP-1) to chromatin (Paro & Hogness, 1991).

Another phenomenon similar to the cell-autonomy of the sex determination decision is seen in the homoeotic gene system. Here, the arrangement of the genes in the antennapedia complex (ANT-C) and bithorax complex (BX-C) of homoeotic loci is collinear with the segments of the fly in which they are expressed. It is proposed that this pattern of expression is brought about by a spreading of heterochromatin along the chromosome to an extent determined by the segment of which each cell is part (Gaunt & Singh, 1990). This pattern of heterochromatic spreading is proposed to be locked into position by the action of the *polycomb*-group and *trithorax*-group genes some of which show homology to the proposed NHC's which are involved in PEV (Paro, 1990). Notably, the Polycomb protein itself also contains the chromodomain discussed above (Paro & Hogness, 1991).

Another system which shows a similar two-step initiation of an autoregulatory loop leading to cell autonomous inheritance of a determined state is the maintenance of the lysogenic state by bacteriophage lambda (Ptashne,1987). The first stage is the production of the transcription factor CII (perhaps analogous to *sis-a, sis-a, da and Dk*). CII initiates transcription from the P_{RE} promoter (analogous to the *Sxl* P_E promoter) which drives transcription of the *cI* repressor gene (analogous to *Sxl*) and from P_I which drives transcription from the lysogenic gene *Int*. As well as shutting down all other genes, the CI repressor also maintains transcription of itself by driving transcription from a second promoter P_{RM} (analogous to Sxl protein splicing out the translational stop codon containing exon from its own hnRNA). It is perhaps unsurprising that systems such as these are used (even in such evolutionary divergent systems) to provide maintenance of a determined state.

It is also interesting to note that in the case of CI repressor protein, cooperativity of binding to the adjacent target sequences lends the system a high degree of sensitivity to fairly small changes in protein concentration thus enabling the lytic/lysogenic 'switch' to be 'thrown' in response to small concentration changes; a quality that is essential to the temperate character of 'phage λ (Ptashne, 1987). The HLH nature of Sis-b and Da present us with the possibility that a similar cooperativity may be occurring here, via dimerisation, to enable the two-fold change in dosage of the *Sxl* locus to effect a large increase in the ability of these proteins to drive transcription from P_E.

The next question that presents itself is; what happens after *Sxl*? We know that *tra* and *tra-2* are epistatic to *Sxl* and so we would expect the regulation of these proteins to be dependent upon *Sxl*.

1.2.2.3 The Next Step - *transformer* and *transformer-2*.

tra and *tra-2* mutant alleles which cause XX flies to be transformed into intersexes have no effect on the soma of XY flies (table 1.2), indicating that these genes are only active in the female soma.

In females, both *tra* and *tra-2* are likely to be required throughout development to enhance female functions and repress male characteristics. This is shown by studies involving the introduction of homozygous-mutant cell clones via mitotic recombination, showing that *tra* and *tra-2* are needed until early pupation in order for the correct female pigmentation to be seen in segments 5 and 6 (Baker & Ridge, 1980). These genes are also required for correct female differentiation of the foreleg. However, in this case the requirement ends at around two days before pupariation. It is thought that the different temporal requirements reflect the different rates of cell division in the progenitor cells of these two tissues. The *tra-2* gene has also been shown to function at different times within the same structure to direct different aspects of female differentiation. For example, in the foreleg, the female number of certain bristles is determined by *tra-2* up to a day before the determination of the female-specific structural aspects of the same bristles (Belote & Baker, 1982). This type of observation may represent the interaction of *tra-2* product with other proteins which are temporally restricted.

The cloning of *tra-2* has revealed that it encodes four transcripts which are alternately spliced as shown in figure 1.10 (Mattox & Baker, 1991).

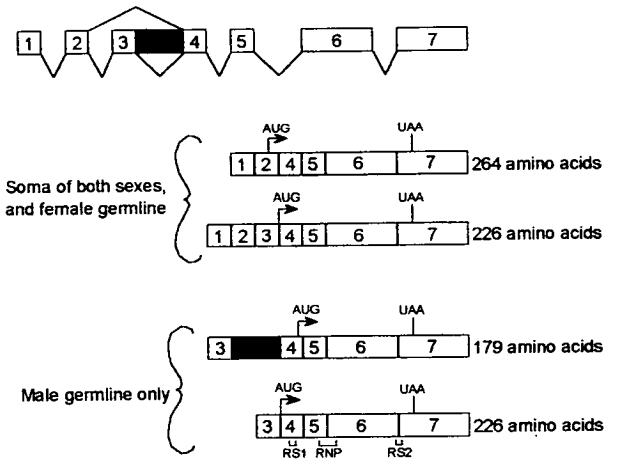


Figure 1.10
Transcripts produced from the *tra-2* locus (taken from MacDougall *et al.*, 1995). Exons are represented by boxes, with the M1 intron being shown as a shaded box. Tissue and sex-specificity, as well as predicted protein sizes, are indicated for each transcript. RNP=ribonucleoprotein domain, RS1 & RS2=arginine/serine-rich motifs.

The *tra-2* transcripts potentially encode proteins with a common C terminus which contain homology to known RNA binding proteins (Goralski *et al.*, 1989). We can see from figure 1.10 that two of the transcripts encode 226 amino acid proteins containing two RS (arginine/serine-rich) domains and one RNP (ribonucleoprotein) domain. The RS domain is common to splicing factors such as U2AF (Zamore *et al.*, 1992; Zhang *et al.*, 1992), SF2/ASF (Ge *et al.*, 1991) and Suppressor of white apricot ($Su(W^a)$; Chou *et al.*, 1987) and is vital for the splicing function of such factors (e.g. Valcárcel *et al.*, 1993; Li & Bingham, 1991). The RNP domain is also common to splicing factors and mediates RNA binding (Amrein *et al.*, 1994). The RS motif is thought to mediate protein-protein interactions between splicing factors (Wu & Maniatis, 1993). The 264 amino acid Tra-2 isoform also contains all three of these motifs. However, the male germline-specific 179 amino acid isoform contains only the RNP domain and RS2. The fact that there are no female-specific somatic transcripts and that no epistasis has been established between *tra* and *tra-2*, together with the fact that *tra-2* has a positive role in spermatogenesis suggests that *tra-2* actually lies on a side branch of the sex determination hierarchy.

The cloning of *tra* has shown that it is indeed part of the sex determination hierarchy, lying directly downstream of *Sxl* (Boggs *et al.*, 1987). As would be expected for a gene regulating determination of tissues, maximal *tra* expression occurs during the pupal stage. Two transcripts are present in the fly as shown in figure 1.11.

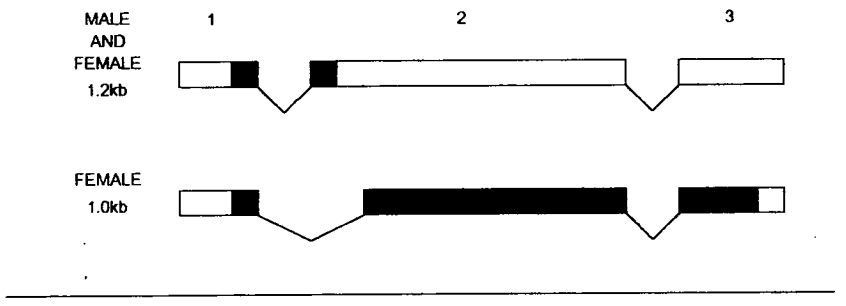


Figure 1.11
Diagram of the sex-specific transcripts produced from the *tra* locus. Exons are indicated by boxes with open reading frames shaded. Sizes and sex-specificities are indicated. See text for details.

It is apparent that both of the *tra* transcripts are initiated from the same promoter. We can also see that *tra* is again subject to sex-specific RNA processing. In the female, the choice of a downstream splice acceptor site prevents the inclusion of a translational stop codon which enables the production of the 211aa active Tra protein. The observation that the area around the non sex-specific splice site contains a uridine octamer sequence indicates that *tra* may be directly under the control of Sxl. Transformation experiments have indeed shown that Sxl is responsible for the sex-specific splicing of *tra* primary RNA and that the uridine octamer is required for this regulation (Sosnowski *et al.*, 1989). This was shown by the use of various constructs containing regions of *tra* genomic DNA, driven by the hsp70 promoter, which were introduced into *tra/tra* flies via germline transformation. Deletion of the non sex-specific splice site led to a degree of Sxl-independent feminisation of the male as would be expected if the function of Sxl is to prevent the use of the deleted splice site. Deletion of the sex-specific splice site had similar effects to the deletion

of the U-octamer, namely female accumulation of unspliced RNA, a lack of female-specific RNA and an inability of this construct to either rescue *tra/tra* females or to transform males.

The mechanism by which *Sxl* blocks the non sex-specific splice site has been shown to be via the antagonism of the essential splicing factor U2AF which binds to the same U-rich sequences as Sxl protein. Sxl protein, however, lacks the splicing 'effector' sequences (arginine-serine repeats, or RS repeats) which are present in U2AF (Zamore *et al.*, 1992; Zhang *et al.*, 1992). If this effector domain is introduced into the Sxl protein, it becomes constitutively active as a splicing factor, causing splicing from the same splice site which it would normally blocks (Valcárcel *et al.*, 1993). A summary of *Sxl*-mediated *tra* sex-specific splicing is shown in figure 1.9(a) and 1.9(b) (middle panels).

The pleiotropic *viriliser* (*vir*) gene has been implicated in *Sxl*-mediated functions (Hilfiker & Nöthiger, 1991). A number of recessive alleles of this locus exist, some of which are lethal to both sexes and some of which cause XX flies to develop as true intersexes. Also, males carrying a constitutive gain-of-function *Sxl* allele which would normally be lethal, are rescued to viable males (i.e. without sex transformation) by the female-lethal vir^{H2} allele. Thus, *vir* is epistatic to *Sxl* and so is likely to lie downstream of *Sxl*, playing a role in both sex determination and dosage compensation. This is supported by the fact that certain *msl* and *mle* mutations partially rescue the lethality of vir^{H2}. *Vir* does, however, lie upstream of *tra* as shown by the rescue of XX flies from vir^{ts}phenotypes, by ectopic expression of the female form of *tra*. XX/vir^{2f} flies (viability achieved via a *mle* null allele) produce the male-specific transcripts of both *tra* and *Sxl*, indicating that Vir may have a role in Sxl-mediated splicing regulation (Hilfiker *et al.*, 1995). *XX/vir2f* flies which also carry a constitutive gain-of-function *Sxl* allele, while viable, are largely male-like, morphologically. These flies splice both *Sxl* and *tra* transcripts predominantly in the male-specific mode. This suggests that Vir is required for Sxl to perform its splicing-modulation function. It is believed that the true intersexual phenotype of

some *vir* alleles reflects an intermediate dosage of gene products from genes below *vir* in the hierarchy, causing both Dsx^M and Dsx^F to be produced in the same cell.

The genes *fl(2)d* and *liz* also appear to be important in regulation of Sxl-mediated splicing (Granadino *et al.*, 1990; Albrecht & Salz, 1993). *XX* flies carrying mutations for either of these genes splice *Sxl* transcript in the male-specific mode. A constitutive gain-of-function *Sxl* allele rescues the mutant phenotype of both of these genes (Steinmann-Zwicky, 1988; Salz, 1992; Granadino *et al.*, 1992) . There is also a *Sxl*-independent role for *fl(2)d* as shown by the non sex-specific lethality of certain alleles (Granadino *et al.*, 1991). This latter function would appear to only be required early on in development as indicated by the fact that adult males carrying a *fl(2)d* temperature sensitive allele are unaffected by temperature shifts. Also, X-ray induced *fl(2)d*-mutant clones are totally viable in the male, when these clones are induced in larvae, indicating that the gene must act before then to perform its vital non sex-specific function.

The next gene in the hierarchy is *dsx*. It is attractive to suppose, bearing in mind the above evidence, that *tra* and *tra-2* products may act upon *dsx* to cause a sex-specific splicing pattern which in turn leads to the production of proteins which implement the female differentiation pathway. In support of such a model for *tra* function, the predicted Tra protein has the RS splicing effector domain seen in U2AF (Boggs, *et al.*, 1987). If the RS domain from the splicing factor suppressor of white apricot ($Su(W^a)$) is replaced by the Tra RS domain, the $Su(W^a)$ protein retains its RS domain-dependent function (Li & Bingham, 1991). This suggests that Tra protein may also function as a splicing factor.

1.2.2.4 *doublesex* and *intersex* - The Sex Determination Effector Genes.

As stated previously, flies mutant for the *dsx* and *ix* genes are true intersexes which is to say each individual cell is of intermediate sexual phenotype. This in itself indicates that these genes are involved in the activation/repression of those genes

which cause differentiation of male and female characteristics. This is further suggested by their epistasis to every other gene of the hierarchy. In other words, it is thought that it is at the stage of *dsx* and *ix* action that determination of the sexual state becomes differentiation of that state.

It is not clear exactly where *ix* fits into the hierarchy. It may lie downstream of *dsx*, as *ix/ix* flies still produce the active female *dsx* transcript and both *ix* and *dsx* are required for correct female development (Baker & Ridge, 1980; Chase & Baker, 1995). However, the possibility that *ix* lies on a side branch cannot be discounted. This model is supported by the proposal that *ix* may be active in males to direct some aspect of pheromone production. This is based on the observation that *ix/ix* males appear to attract other males (Tompkins, 1986). However, *ix* male-specific function has not been shown conclusively.

The fact that null *dsx* alleles affect both sexes while certain other alleles affect only males or females, tells us that *dsx* must be differentially active in both sexes. The cloning of the gene has enabled a developmental profile of transcripts produced from *dsx* that is in agreement with this mode of action (Baker & Wolfner, 1988). This developmental profile is shown in table 1.3.

	Embryonic & Larval Stages.	Pupa	Adult Fly
MALE	1.65kb 2.8kb	2.9kb 3.9kb	2.9kb 0.7kb 3.9kb
FEMALE	1.65kb 2.8kb	3.5kb	3.5kb

Table 1.3
Developmental profile of *dsx* transcripts. Sizes of *dsx* transcripts detected throughout development are shown in kilobases.

The 1.65kb and 2.8kb transcripts share 3' and 5' ends but differ in the intervening sequence, showing that they are derived from the same primary RNA. The region covered by these transcripts represents a minimum of 40kb of genomic DNA (Baker & Wolfner, 1988). This is comparable to the 23kb *Sxl* locus but is much greater than the very small 2kb *tra* gene (Boggs *et al.*, 1987).

The cloning of *dsx* cDNA's representing the 3.9kb and 3.5kb transcripts (Burtis & Baker, 1989) has demonstrated how the *dsx* gene can be differentially active in both sexes (figure 1.12). The transcripts shown in figure 1.12 are differentially spliced and polyadenylated but are both capable of producing large proteins. Hence it would be quite feasible for there to be differential activity in both sexes.

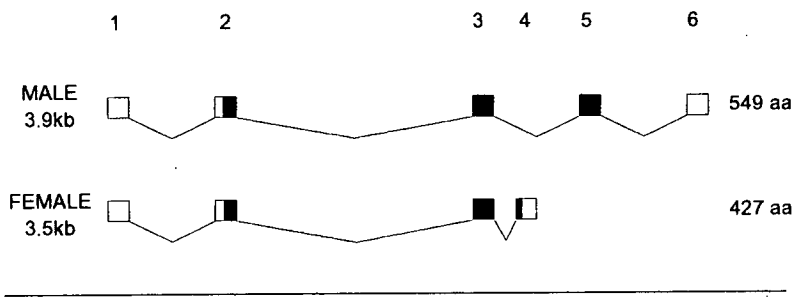


Figure 1.12
Intron/exon structure of the two major adult sex-specific *dsx* transcripts. Exons are shown as boxes, with open reading frames shaded. Transcript and predicted protein sizes are indicated.

It has recently been shown that there is a common region of Dsx^M and Dsx^F which is responsible for binding to the regulatory regions of downstream genes which are regulated directly by Dsx (Erdman & Burtis, 1993). The common N-terminal domain of these proteins carries a putative α-helical domain which is required for binding activity as shown by gel-shift assays with Dsx proteins lacking this region. Site directed mutagenesis has shown that certain cysteine residues next to this region are also critical for DNA binding and that this binding is EDTA sensitive. Also, spectrophotometric detection has shown that observed binding of divalent metal cations is reduced when these residues are mutant. The binding site has been classed

as a novel zinc finger type as it bears no homology to previously identified zinc finger motifs.

It would be a natural progression to suppose that the production of the Dsx^F transcript is under the control of the Tra and Tra-2 proteins. In fact this turns out to be the case. Germline transformation using the female-specific *tra* cDNA fused to the hsp70 promoter transforms the male soma towards that of the female (Boggs *et al.*, 1987). Direct evidence has been published that shows that *tra* and *tra2* cause the female-specific transcript to be produced (Hoshijima *et al.*, 1991; Ryner & Baker, 1991). Plasmid containing *dsx* genomic DNA from exon 3 to exon 5 (see figure 1.12) was transfected into *Drosophila* culture cells either alone or together with hsp70-driven female-specific *tra* cDNA or with hsp70-driven *tra-2* cDNA, or with both (Hoshijima *et al.*, 1991). It was observed that if Tra and Tra-2 proteins were not present, male-specific *dsx* RNA was produced and practically no female-specific transcript was detected. If Tra or Tra-2 was present then much more female-specific *dsx* RNA was produced. Finally, if Tra and Tra-2 were present then only female-specific *dsx* RNA was produced. A construct containing the region from exon 3 to exon 4, but not containing the female-specific polyadenylation signal, was capable of being directed by Tra and Tra-2 to utilise the female-specific splice acceptor. However, a construct lacking this splice site was spliced in a Tra/Tra-2-independent fashion. Thus, the only possible model to account for this is that Tra and Tra-2 act at the female-specific acceptor splice site, to positively promote the usage of this site.

Transgenic studies have shown that all three Tra-2 protein isoforms (figure 1.10) are capable of regulating the splicing of *dsx*, with the 226 amino acid and 264 amino acid proteins being the most efficient (Mattox *et al.*, 1996). The transcripts encoding all three isoforms, under the control of their cognate promoter elements, were introduced into *tra-2/tra-2* females via germline transformation and subsequent crosses. *XX/tra-2* flies expressing either of the two larger Tra-2 isoforms developed as normal females and expressed the female-specific *dsx* transcript. The smaller 179

amino acid isoform, which lacks the RS1 motif, was only capable of partially rescuing the pseudomale phenotype of *XX/tra-2* flies and both male-specific and female-specific *dsx* transcripts were produced.

Six 13-nucleotide repeats (*dsx* 13-nt repeats) contained in exon 4 have been identified as being important for Tra/Tra-2-mediated *dsx* splicing. Deletion of these repeats causes a loss of female-specific product in the cotransfection system described above (Hoshijima *et al.*, 1991). Also, substitution of some of the non-canonical purines present in the polypyrimidine (polyY) tract of the female-specific acceptor site causes female-specific splicing independent of Tra and Tra-2. This indicates that this site is not used in males because of its non-standard polypyrimidine stretch and that in females Tra and Tra-2 act to stabilise the splicing apparatus at this site and thus promote its use. Tra2 protein binds to RNA containing one of the *dsx* 13-nt repeats and a 540bp sequence containing the six 13-nt repeats is necessary and sufficient to direct Tra/Tra-2-mediated splicing of the *dsx* male-specific splice acceptor (Ryner & Baker, 1991).

In addition, arginine-serine repeat-containing splicing factors can be recruited by Tra and Tra-2 to the region containing the 13-nt repeats (Tian & Maniatis, 1993), and Tra and Tra-2 proteins have been shown to physically interact both with each other and with the splicing factor, SF2 (Amrien *et al.*, 1994). The six 13-nt repeats of the *dsx* gene have been shown to interact synergistically with a purine-rich element (purine rich enhancer, or PRE) in the same region, to promote use of the female-specific splice site (Hedley & Maniatis, 1991; Lynch & Maniatis, 1995).

A summary of the RNA splicing regulation of the *Sxl*, *tra* and *dsx* genes is shown in figure 1.9 and the whole sex determination hierarchy is summarised in figure 1.13. This hierarchy controls the sex determination of the soma. However, the determination of the germline does not require the action of all these genes and it is this tissue which is discussed next.

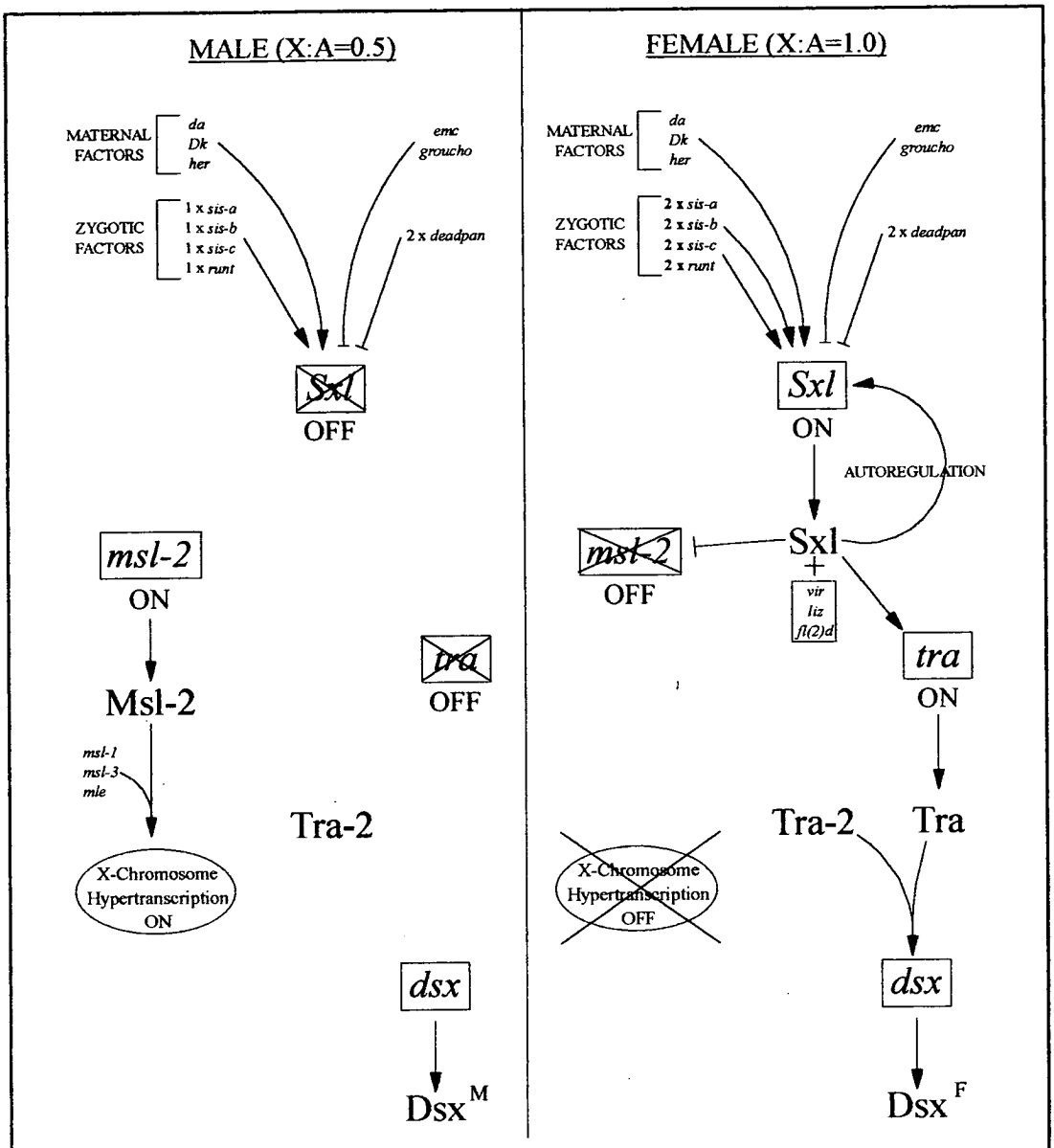


Figure 1.13
Summary of the sex determination hierarchy of *Drosophila*. See figure 1.9 for mechanistic details.

Activation of *Sxl* is dependent upon the X:A ratio, due to the action of X-linked transcriptional repressors (numerators) in balance with autosomal transcriptional repressors (denominators).

In males, *Sxl* remains inactive and has no function. The *msl-2* transcript is spliced to produce active mRNA. Msl-1, Msl-2, Msl-3 and Mle act in a single pathway to promote generalised hypertranscription of the X chromosome. The *dsx* primary RNA is spliced in the default mode to produce Dsx^M protein.

In females, *Sxl* is activated and is locked 'ON' by positive autoregulation. Sxl protein acts upon the *msl-2* and *tra* primary transcripts to modulate their splicing, such that an inactive *msl-2* mRNA and an active *tra* mRNA are produced. Tra and Tra-2 proteins act in concert to modulate the splicing of the *dsx* primary transcript, such that a female-specific Dsx protein, Dsx^F, is produced.

1.3 SEX DETERMINATION OF THE GERMLINE

1.3.1 CELL-AUTONOMOUS AND INDUCTIVE FACTORS

Initial investigations into sex determination of germ cells were done by pole cell transplantation between male and female embryos (Van Deusen, 1976). It was seen that XX gametes only developed in XX gonads and XY gametes only developed in XY gonads. This indicates that germ cells cannot develop in the gonad of the opposite sex. This means that the pole cells must, at the time of transplantation, be at least partly determined by their chromosomal sex to become either spermatogenic or oogenic, since they cannot be subverted to the developmental pathway of the opposite sex by the presence of the somatic gonad tissue of that sex. It also indicates that there must be some communication between the germ cells and the somatic cells of the gonad which allows the germ cells to differentiate male gonad somatic cells from those of the female. The death of germ cells transplanted into embryos of the inappropriate sex may to some extent be due to inappropriate X chromosome dosage . In support of this, the X-hypertranscription gene *mle* has been shown to have some function in the male germline (Bachiller & Sánchez, 1986). Thus, unlike somatic sex determination which involves purely cell autonomous decisions, sex determination in the germline involves a mixture of cell autonomous and inductive factors.

The autonomous nature of XY germ cells is shown by pole cell transplantations into host embryos which have been genetically manipulated to contain no germline. Both XY and XO pole cells appear to enter spermatogenesis in the ovary of an XX host, as characterised by the large nuclei, nucleoli and mitochondrial clusters typical of spermatocytes (Steinmann-Zwicky *et al.*, 1989). Crystal structures are seen in the abortive XO cells. When XX pole cells are transplanted into a germline-deficient XY embryo they again appear to enter spermatogenesis, forming abortive sperm-like cells with the same crystal structures. Initially, this evidence was taken to indicate that male and female pole cells are autonomous to the extent that they exhibit different 'competence' to be directed down the spermatogenic or oogenic pathways,

this competence being controlled by the X:A ratio. This was indicated by the fact that while 1X:2A (X:A=0.5) germ cells can only enter spermatogenesis, 2X:3A (X:A=0.67) germ cells appeared to be able to enter either spermatogenesis or oogenesis (Nöthiger *et al.*, 1989). However, more recent experiments have required a re-thinking of this hypothesis, as described below. This work centres on studies involving two genes which have roles in female germ cell development; *ovarian tumour* (*otu*) and *ovo*.

Mutant alleles of the *ovo* gene only affect females. Germ cells are sensitive to a lack of *ovo* product at around the late blastoderm-early gastrula stage and will die at this stage in *ovo* mutant flies, giving rise to empty ovaries (Oliver *et al.*, 1987). Chimeric flies show that this reflects a cell autonomous requirement for *ovo* (Perrimon & Gans, 1983). Different phenotypes have been observed for different *ovo* alleles, indicating that *ovo* is not only involved in sex determination. Some allele combinations do produce male-like germ cells but it is more likely that *ovo* is involved in modulating more general developmental functions in the oocyte, since mutations in sex transforming genes do not generally cause such severe death of germ cells as *ovo* alleles (Oliver *et al.*, 1990). The gene has been cloned and has been found to encode a putative transcription factor containing four zinc fingers (Mével-Ninio *et al.*, 1991).

Mutant alleles of the *otu* gene produce very similar phenotypes to *ovo* mutations (Pauli *et al.*, 1993). A set of recent experiments on the *otu* and *ovo* genes have provided further insights into the nature of a female-specific somatic signal required for development of XX germ cells (Nagoshi *et al.*, 1995). Several lines of evidence indicate that the transduction of the female somatic inductive signal is, at least in part, mediated by *otu*. When *XY* flies are transformed into pseudofemales by ectopic *tra* expression, the gonads of the flies can by thought of as a 'pseudo-ovary', containing genetically male germ cells surrounded by genetically female gonadal somatic cells. The follicle cells of such 'pseudo-ovaries' appear essentially wild-type but the germ cells are highly abnormal, having egg chambers filled with large

quantities of small, tumourous, egg cysts (Nagoshi *et al.*, 1995). When the germ cells of a pseudo-ovary are mutant for a severe *otu* allele, no germ cells are observed in the egg chambers. If the same *otu* allele is complemented by ectopic *otu* expression, the egg chambers are again seen to be filled with egg cysts. In other words, placing a XY germ cell in a female somatic environment imposes upon it a requirement for *otu*. The same requirement is not seen for *ovo*, with egg cysts being produced in pseudo-ovaries regardless of the integrity of the *ovo* locus. In the converse set of experiments, XX germ cells developing in 'pseudo-testes' were seen to require both the *otu* and *ovo* genes for proliferation to occur. Thus, the XX germ cells maintain a cell autonomous requirement for *otu* and *ovo*, irrespective of the sex of the surrounding soma. On the strength of the above evidence, Nagoshi *et al.*, 1995, propose that the female somatic signal induces *otu* expression in the XX germ cells which, together with cell-autonomous Ovo, allows the cells to proliferate and develop.

This evidence is in conflict with previous pole cell transplantation experiments which showed that XY pole cells were not 'competent' to enter oogenesis (see above). These pole cell transplantation experiments suggested that XY germ cells developed into spermatocytes, whether they developed in a testis or in an ovary (Steinmann-Zwicky *et al.*, 1989). The XY germ cells in a pseudo-ovary were morphologically very similar to the cells that were classified as spermatocytes in the pole cell transplantation experiments (Nagoshi *et al.*, 1995). However, since the female germline-required gene *otu* is necessary for production of these cells in the psuedo-ovary, it seems that they are undergoing some aspects of oogenic development. It appears that the problem is one of classification. Germline sex determination is not controlled via a linear hierarchy in the same way as somatic sex determination. It is much more likely to require a number of parallel pathways to achieve correct gametogenesis. Therefore, mutation of a single gene may not result in a simple 'switch' between male or female developmental pathways, but rather some dimorphic phenotype which is a mixture of male and female characteristics. Thus, the cells that were classified as spermatocytes in the pole cell transplantation

experiments, may in fact be neither spermatocytes nor oocytes, but a mixture of the two.

Nagoshi *et al.*, 1995, propose that both male and female germ cells are subject to sex-specific signals from the somatic cells of the gonad. In XX cells, a follicle cell signal would result in activation of *otu* which, together with *ovo*, would mediate certain oogenic processes. It is proposed that an analogous system operates in the testis, such that an as yet unidentified testis-specific *otu*-counterpart would be activated in response to a male-specific signal from somatic testis tissue. This would account for all of the above observations. A XY germ cell in an ovary, or pseudo-ovary would be subject to the inappropriate female-specific signal and *otu* would be activated in the cell. This would result in *otu*-mediated processes being activated in conjunction with cell autonomous spermatogenic processes which may result in the abortive "spermatocytes" observed. The converse argument can be applied to XX germ cells in a testis, or pseudo-testis.

1.3.2 THE FEMALE INDUCTIVE SIGNAL- PRODUCTION AND TARGETS

At present, nothing is known about the possible origins or targets of the putative male-specific signal. However, the female-specific somatic signal is under the control of the sex determination hierarchy in the follicle cells, as shown by the following evidence. XX germ cells which are mutant for each of the somatic hierarchy genes; *tra* , *tra-2* , *dsx* and *ix* develop correctly when introduced into a wild type XX embryo (Marsh & Wieschaus, 1978; Schüpbach, 1982). Thus, these genes are not required cell-autonomously for oogenesis. However, when XX pole cells were transplanted into an XY host which was feminised by ectopic expression of a female-specific *tra* cDNA, it was observed that the XX pole cells entered oogenesis and indeed formed complete eggs (Steinmann-Zwicky & Niederer, unpublished, in Steinmann-Zwicky, 1992). In addition, alteration of *tra* or *dsx* expression can affect germ cell morphology (Steinmann-Zwicky, 1994).

This model is complicated by the observation that in *tra/tra* or *tra-2/tra-2* pseudomales, abortive oocytes as well as abortive and immotile sperm can be found (Nöthiger *et al.*, 1989). If a feminising inductive factor is produced via the somatic hierarchy, then how can oogenesis occur in *tra/tra* or *tra2/tra2* flies? It has been proposed that some initial signal under the control of the sex determination hierarchy directs the XX pole cells down the oogenic pathway very early in development. However, these cells can still revert to spermatogenesis unless the oogenic pathway is confirmed later on in development by a further hierarchy-independent signal from the surrounding soma. Similarly, pole cells which have started down the spermatogenic pathway can be subverted by the presence of the later signal to follow the later stages of the oogenic pathway. This model is based on the morphology of XX pseudomale pole cells throughout development, as described below (Steinmann-Zwicky, 1992).

Up until the first larval instar, male and female pole cells are indistinguishable. Upon hatching, however, the male gonad is much larger than the female one, containing more germ cells. Thus, by this stage the initial feminising signal must have played its role, as the pole cells have entered one or other of the developmental pathways. In the second and third larval instars, and during metamorphosis, spermatogenic structures and oogenic structures, as well as degenerating cells, are seen. The presence of these structures shows that complete determination has not taken place by the first larval instar and so further signals must be required. Examination of the development of XX germ cells in pseudomales lacking the sex determination function of *Sxl* shows that *Sxl* is somatically required for the production of later oocyte structures such as nurse cells, in these flies (Nöthiger *et al.*, 1989). Thus, it seems likely that *Sxl* controls the production of both the early and late feminising signals but through different pathways, such that the early signal is somatic hierarchy-dependent while the late signal is not.

We can now ask the question; what is the effect that the early hierarchy-dependent signal has upon XX germ cells? It seems likely that the action of the signal is to

direct *Sxl* to produce germline-specific *Sxl* transcripts. There is a number of pieces of evidence that point to this model. Firstly, there is a delay between the appearance of *Sxl* protein in the soma and its detection in the pole cells, as might be expected if signaling was required between the two tissues (Bopp *et al.*, 1991). Secondly, when *X/X, Sxl/Sxl* germ cells develop in an XX host, any oocytes formed are cystic in nature and even spermatogenic cells are produced (Schüpbach, 1985). Conversely, XX germ cells carrying a constitutive *Sxl* gain-of-function allele enter oogenesis even when transplanted into a male host (Steinmann-Zwicky *et al.*, 1989). The germline *Sxl* product is different from the somatic *Sxl* product, as indicated by the presence of *Sxl* alleles which cause cystic ovaries but do not otherwise affect the development of the fly, and by the fact that the 1.9kb and one of the 3.3kb *Sxl* late female-specific transcripts are germline-specific (Samuels *et al.*, 1991). The 4.2kb and other 3.3kb transcripts are found both in the soma and the germline. The tissue specificity of the two 3.3kb transcripts was determined by comparing abundance in wild type and germline-deficient strains.

The *orb* locus encodes RNA-binding proteins and produces male and female germline-specific transcripts which are alternately spliced (Lantz *et al.*, 1992). Analysis of the splicing patterns of *Sxl* and *orb* in various mutant backgrounds has indicated that, while *tra* and *dsx* are required for certain aspects of oogenic development, only *tra-2* is required in the soma for the production of the female-specific somatic signal which directs the female germ cells into oogenesis (Horabin *et al.*, 1995). In XX flies which are mutant for *dsx*, the majority of *Sxl* and *orb* germline-specific transcripts are of the female-specific class and Sxl protein is clearly detectable. However, XX flies which carry a *tra-2* null allele exhibit virtually exclusive male-specific patterns of *orb* and *Sxl* expression and very little Sxl protein is detected, if any. Surprisingly, *tra* does not appear to be required for this *tra-2*-mediated function, since XX flies carrying some combinations of *tra* alleles exhibit mostly female-specific expression patterns of *Sxl* and *orb*, with Sxl protein being produced. Thus, it may be that Tra-2 can act in conjunction with an, as yet unidentified, cofactor other than Tra to regulate the production of the female-specific

somatic inductive signal. It appears that, while *tra* and *dsx* are required in the female soma to direct certain aspects of female germ cell development, neither gene is involved in production of the signal which sets the germ cells upon the oogenic pathway.

1.3.3 ACTIVATION OF *SXL* IN THE FEMALE GERMLINE

It has been shown that while *da* is not required, the gene *liz* is required for oogenesis to take place (Saltz, 1992). Female flies homozygous-mutant for *liz* develop cyst-filled ovaries. This phenotype is completely rescued by a gain-of-function *Sxl* allele and reflects an autonomous requirement for *liz*, as female flies with *liz/liz* germ cells lay no eggs. Therefore, it would appear that *liz* is required cell-autonomously for activation of *Sxl* in the germline and is probably involved in splicing regulation as previously suggested. The gene *fl(2)d* is also required for *Sxl* activation in the germline (Steinmann-Zwicky, 1993). Also, the *otu* gene has been implicated in *Sxl* activation, since some *otu* alleles produce ovarian tumours typical of *Sxl* germline-specific alleles (Pauli *et al.*, 1993). Sxl protein is not produced in ovaries in flies carrying *liz* or *otu* mutations (Bopp *et al.*, 1993).

Oliver *et al.*, 1993, showed that several genes which cause ovarian tumours when mutant, are required cell-autonomously in the the female germline for female-specific splicing of the *Sxl* transcripts. These include the *otu*, *ovo* and *liz* loci. One other such gene is the *fused* locus, which encodes a serine/threonine kinase and as such may be involved in transducing the follicle cell signal into the female germline-specific *Sxl* splicing pattern.

Germline clones which are mutant for the genes *sis-a*, *sis-b* and *runt*, still produce oocytes in the female (Steinmann-Zwicky, 1993; Granadino *et al.*, 1993). In the soma, these mutations result in lethality due to an inability to activate *Sxl*. This could mean that *sis-a*, *sis-b* and *runt* play no role in activation of *Sxl* in the germline or that *Sxl* expression is already set up at the time of clone induction or transplantation.

The *bag of marbles* gene is required for gametogenesis in both sexes (McKearin & Spradling, 1990). Mutant alleles of the gene result in undifferentiated cyst-like cells in ovaries and testes. In oocytes, Sxl protein shows an abnormal localisation pattern in these mutants (Bopp *et al.*, 1993).

If the activation of *Sxl* is the only effect which the feminising signal produces in the female, then we would expect XY pole cells to enter oogenesis if they carried a constitutive *Sxl* allele. This is not the case. XY germ cells remain spermatogenic even though they carry either of the constitutive gain-of-function *Sxl* alleles, $Sxl^{M\#1}$ or $Sxl^{M\#4}$, and develop in an XX host (Steinmann-Zwicky *et al.*, 1989). How can this be explained? Either there is some other cell autonomous property of XX or XY pole cells which is *Sxl*-independent and limits the inductive competence of these cells, or the *Sxl* alleles do not direct sufficient *Sxl* production in the XY pole cells. There is some evidence for the latter explanation as it is apparent that in XY flies not all somatic cells are feminised by the $Sxl^{M\#1}$ allele. However, $Sxl^{M\#4}$ is known to express *Sxl* constitutively in somatic XX and XY cells and in XX germ cells (Steinmann-Zwicky, 1993). Therefore, it appears that while *Sxl* activation is critical to female germ cell development, there are a number of other cell-autonomous pathways acting in parallel which are requires for correct oogenesis to occur. In support of this model, several of the genes involved in female germline development can be categorised according to their phenotypes. Mutant alleles of *otu* and *ovo* cause a range of phenotypes ranging from complete loss of female germline, to sexual transformations of the germ cells, while *Sxl* or *liz* mutations cause sexual transformations, but have no effect on germ cell viability (Oliver *et al.*, 1993). This indicates that *otu* and *ovo* are involved in a viability pathway as well as the *Sxl* activation pathway which leads to sexual transformations.

The above evidence shows that *Sxl* function in the germline is different from its function in somatic cells, in that *Sxl* does not appear to control a hierarchy of germ cell sex-determining genes but is rather a part of a pathway of genes which is only one of a number of parallel pathways required for correct oogenesis. Further support

for this comes from studies of germline markers in germ cells which have perturbed *Sxl* function (Bae *et al.*, 1994; Horabin *et al.*, 1995). For example, the *orb* female-specific splicing pattern is observed exclusively in ovaries, even when they lack any detectable Sxl protein (Horabin *et al.*, 1995).

1.3.4 FUNCTIONS OF *TRA-2* IN THE MALE GERMLINE

As well as a female-specific somatic requirement for *tra-2*, this gene also functions cell-autonomously in the male germline, since non-functional sperm are produced in *tra-2*-mutant flies (Belote & Baker, 1983). Indeed, most abundant expression of *tra-2* is seen in this tissue and two male germline-specific transcripts are produced by the *tra-2* locus as shown in figure 1.10 (Mattox & Baker, 1991). In wild type flies, the removal of the M1 intron is normally an inefficient process such that the concentration of the M1-containing transcript is higher than that of the the completely spliced transcript. In male flies carrying a *tra-2* null mutation, removal of the M1 intron occurs to completion (Mattox & Baker, 1991). Mutation of the M1 splice sites increases the levels of M1-containing transcripts. It has been shown by ectopic expression of M1 intron-containing cDNA under the control of the hsp70 promoter that this transcript is not sufficient to produce developed sperm (Mattox & Baker, 1991). Therefore, it would appear that Tra-2 protein in the male germline acts to increase levels of M1 intron-containing transcript. The fact that this transcript has no observable function, suggests that this acts as a type of negative feedback mechanism to limit the levels of active Tra-2 in the male germline. In support of this, Tra-2 ectopic transgenic expression experiments show that, of the two Tra-2 isoforms present in the germline, only the larger, 226 amino acid, protein is capable of promoting correct spermatogenesis in *XY/tra-2* flies (Mattox *et al.*, 1996). Thus, the 179 amino acid protein produced from the M1-containing transcript has no function in spermatogenesis. As well as restoring correct spermatogenesis to *XY/tra-2* flies, the 226 amino acid Tra-2 protein also directs the production of M1-containing *tra-2* transcript and testis-specific *exu* transcript (Mattox *et al.*, 1996). It is interesting to note that sequences with similarity to the 13-nt repeats which are

required for Tra/Tra-2-mediated splicing of the *dsx* transcript have also been found in both *tra-2* and *exu* cDNAs (Mattox & Hazelrigg, pers. comm.).

1.3.5 SUMMARY

The available evidence suggests that both male and female germ cells require an inductive signal from the somatic component of the gonad which, along with cell autonomous processes, is required for correct gametogenesis. It is not clear what genes are involved in these processes in male germ cells, but a model is emerging for female germ cells (figure 1.14). An inductive signal, under the control of *tra-2*, is required from the follicle cells for correct oogenesis. This signal results in germline-specific splicing of *Sxl* transcripts to enable production of active Sxl protein. The Fused, Liz, Otu and Ovo proteins are all required in the germline for *Sxl* activation, along with a X:A ratio of 1.0. The Fused serine/threonine kinase may be involved of the transduction of the somatic signal and might modulate the activity of transcription factors, such as Ovo. The successful transduction of the inductive signal is dependent upon activation of the *otu* gene. As well as promoting *Sxl* activation, the Otu and Ovo proteins appear to be involved in a cell-autonomous pathway which is essential for female germ cell viability.

A recent screen for modifiers of the ovo^D dominant allele has identified at least 4 regions containing ovo^D suppressors and at least 6 regions containing ovo^D enhancers (Pauli *et al.*, 1995). The authors estimate that these regions contain around 20 *ovo*-modifier loci. Further studies of these loci may enable the isolation of factors important in germline sex determination and lead to further elucidation of the processes involved.

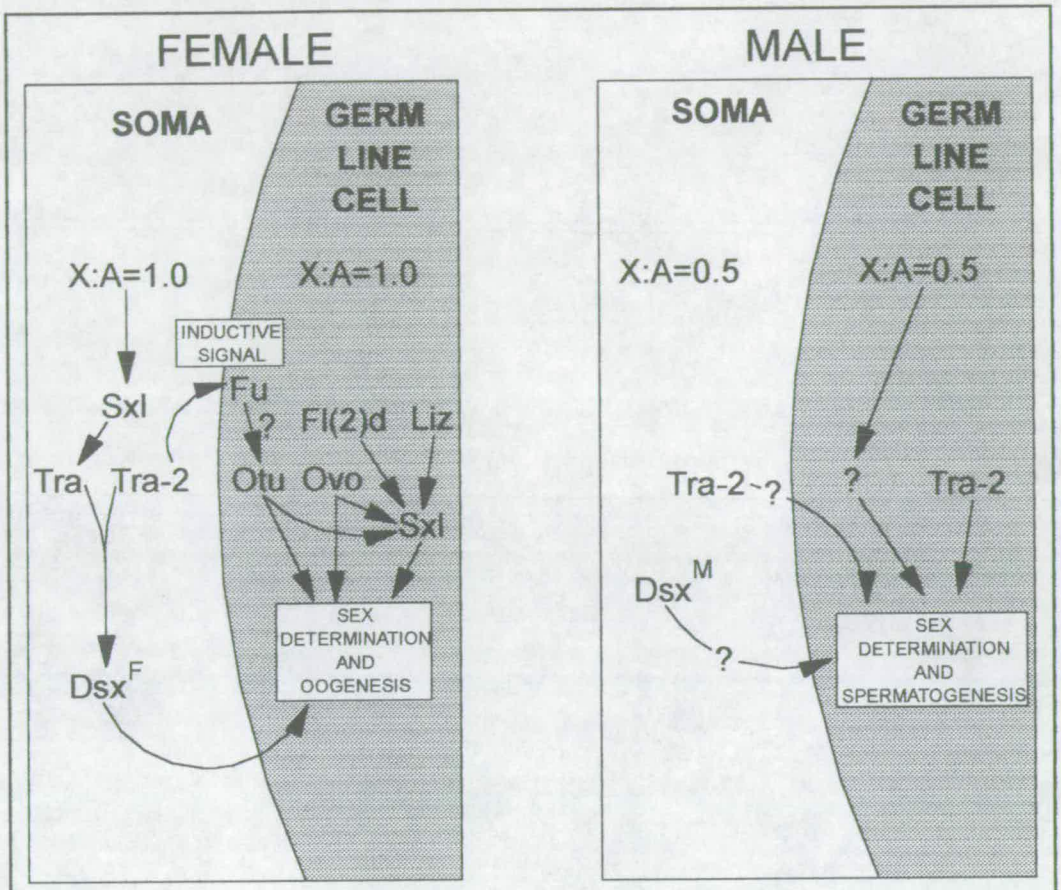


Figure 1.14

Diagram of the factors known to play a part in germline sex determination and differentiation in *Drosophila*. In the female, an inductive signal is required for pole cells to be determined as female. The *tra-2* gene is required in the soma for this signal to be sent. Neither *tra* nor *dsx* appear to be required. Tra-2 may act in concert with an, as yet unidentified, cofactor other than Tra. The S/T kinase encoded by the *fused* gene is required cell-autonomously in the female germline and may be involved in transducing the somatic signal. The *otu* gene is required for pole cells to respond to their somatic environment and may lie downstream of the Fu kinase. The *Sxl* gene is only activated in female germ cells and the genes *otu*, *ovo*, *liz* and *fl(2)d* are required for this activation. The *otu* and *ovo* genes are involved in oogenic functions other than sex determination, as is the somatic *dsx* gene. In male germ cells, *tra-2* is required cell-autonomously for correct spermatogenesis. There may also be a requirement for somatic induction under hierarchy control, but it is not known which genes control this putative signal.

1.4 FACTORS INVOLVED IN SOMATIC SEX DIFFERENTIATION

A great deal is known about the genes and processes which are involved in determining the sex of a cell in the developing embryo of *Drosophila*. Much less is known at the molecular level about how this decision is transduced into a sexually dimorphic phenotype. To date, the only non-gonadal sex differentiation genes to be cloned are the *yolk protein* genes.

1.4.1 THE *YOLK PROTEIN* GENES

There are three *yolk protein* genes (*yp1*, *yp2* and *yp3*) and all are X-linked. The *yp1* and *yp2* genes are divergently transcribed as shown in figure 1.15. The *yp3* gene is situated over 1000kb away from *yp1* and *yp2*.

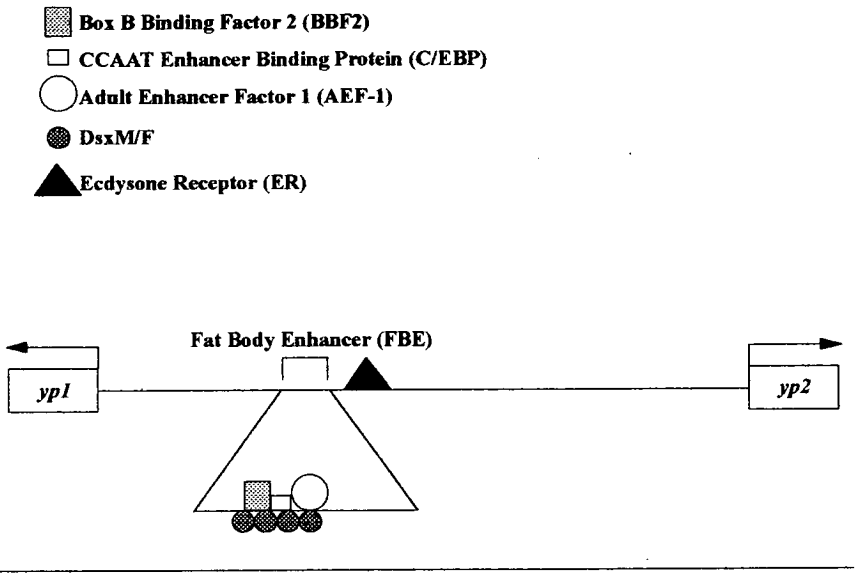


Figure 1.15
Factors involved in female fat body-specific expression of *yp1* and *yp2*. Protein binding sites, as shown by footprint analysis, are indicated. Directions of *yp* transcription are shown.

The regions which have so far been identified as important in the regulation of *yp1* and *yp2* are indicated in figure 1.15 (reviewed in Bownes, 1994). The differential

regulation of the *yp's* in the female fat body and follicle cells is dependent upon separable regions within the intergenic region and in the first exon of *yp2*. Correct levels of follicle cell-specific *yp* expression are dependent upon the elements OE1 and OE2 which act synergistically to confer this tissue-specific expression pattern (Logan *et al.*, 1989; Logan & Wensink, 1990). A 125bp orientation-dependent region named the fat body enhancer (FBE) was initially thought to be sufficient and essential for expression of the *yp's* in the fat body (Garabedian *et al.*, 1986). This is not in fact the case, as shown by *Adh* reporter gene constructs driven by regions of the intergenic spacer (Abrahamsen *et al.*, 1993). The *yp2* gene and 887bp of upstream DNA is sufficient to confer female fat body expression upon the reporter gene. Similarly, sequences upstream and downstream of, but not including, the FBE also confer this tissue and sex-specificity. Therefore, it is apparent that no one sequence is solely responsible for the fat body and sex-specific expression of *yp1* and *yp2*. It has been demonstrated that sequences within 705bp of *yp3* upstream DNA are sufficient to drive tissue and sex-specific expression of this gene (Liddell & Bownes, 1991) and that this region can be subdivided into separate elements which control either fat body-specific or ovary-specific expression (Ronaldson & Bownes, 1995).

Footprinting and binding studies have shown that Dsx^M and Dsx^F bind to the FBE in an identical fashion, as would be expected from the shared binding sites within the proteins (Burtis *et al.*, 1991). Computer sequence analysis has shown that there are four Dsx binding sites within this region and 32 possible sites within the whole intergenic region (Abrahamsen *et al.*, 1993).

The fat body of *Drosophila* is thought to be a homologous tissue to mammalian liver. For this reason, proteins which control liver-specific expression of the mammalian *Adh* gene were used in FBE footprinting experiments (Abel *et al.*, 1992; Falb & Maniatis, 1992). Footprints of the AEF-1, C/EBP and BBP-2 proteins overlap the Dsx footprints as shown in figure 1.15. C/EBP is a transcriptional enhancer of the *Adh* gene in rat hepatocytes. AEF-1 is a repressor of C/EBP and so may have this

same function in *yp* expression. AEF-1 activates *Adh* expression in the fat body of *Drosophila*.

1.4.2 DSX AS A GLOBAL REPRESSOR OF SEX DIFFERENTIATION GENES

Null *dsx* mutations cause flies to develop as intersexes (Baker & Ridge, 1980). The phenotype of these flies appears to result from simultaneous expression of both male and female sex differentiation. This suggests that the Dsx^M and Dsx^F proteins act purely as repressors of inappropriate sex differentiation genes.

The fact that *XY/dsx* null mutants express the *yp's* at a high level in the fat body suggests that Dsx^M is a *yp* gene repressor and that Dsx^F has no role in this system (Bownes & Nöthiger, 1981). The dominant dsx^D allele constitutively expresses the male form of Dsx protein, Dsx^M. XX/dsx^D flies develop as pseudomales which exhibit no *yp* expression in the fat body, indicating that Dsx^M is a repressor of the *yp* genes.

A purely repressive role for Dsx is further indicated by the fact that *dsx* null intersexes have mosaic genitals containing some male and some female structures, showing that both genital primordia have developed to some extent (Baker & Ridge, 1980). *tra* and *tra-2* are required throughout development for the repression of the male genital primordium (Wieschaus & Nöthiger, 1982; Belote & Baker, 1982; Epper & Bryant, 1983). Taken together these data suggest that Dsx^M represses the female genital primordium and Dsx^F represses the male genital primordium. There is however evidence that both Dsx proteins also have positive roles in activating transcription.

Ectopic expression of a hsp70-driven construct containing a cDNA encoding the Dsx^M protein leads to three novel phenotypes, indicating a positive role for Dsx^M (Jursnich & Burtis, 1993). Firstly, an abnormal pigmentation was seen in the third instar larva which was proposed to be an indication of a positive role for Dsx^M in male-specific abdominal pigmentation. This is supported by the observation that a

reduced level of adult pigmentation is seen in flies carrying *dsx* null mutations. The second phenotype is seen in the foreleg of the fly. The foreleg of *Drosophila* is sexually dimorphic in a number of ways. Males have more gustatory receptors than females and a male-specific row of large bristles known as the sex combs. It was observed that ectopic expression of the Dsx^M protein caused a large number of bristles on all the legs of both male and females to be transformed towards a sex comb-like phenotype. This was taken as evidence for a positive role for Dsx^M in this process also.

The results from the ectopic expression of Dsx^M, although compelling, can be explained within a model that only allows Dsx^M a repressive function. If we imagine a group of male-specifically expressed genes which control the development of a male-specific characteristic such as the sex comb or abdominal pigmentation, then these genes would only be active in the tissue in which they are required. What inactivates these genes in the rest of the fly? It may be that some high-activity repressive factor which is expressed under the control of the homoeotic loci in a spatially restricted manner is responsible for this. The tissue in which the male-specifically expressed genes are required, would be instructed not to produce this repressor. In the equivalent tissue in the female, Dsx^F would repress the male specifically-expressed genes so that the absence of the homoeotic-controlled transcription factor would not enable their expression. If ectopic Dsx^M is present, it could compete with the homoeotic-controlled repressor (and with Dsx^F) allowing some expression of male-specifically expressed genes throughout the fly and thus giving rise to ectopic male-like structures. This type of model may provide an explanation for the third observed phenotype seen in these studies, which is increased lethality. Such lethality may be caused by the extensive ectopic differentiation of male characteristics in a largely female background.

Further evidence for a positive role for *dsx* comes from the observation that Dsx^F can cause a four-fold increase in transcription of a lacZ reporter gene which is driven by sequences from the *yp* FBE (Coschigano & Wensink, 1993). The integrity of the *dsx*

binding sites in the FBE was required for this to take place. This represents a four-fold increase in expression over and above the basal level of expression exhibited by the same construct when it is introduced into flies which are homozygous for a *dsx* null mutation. This would indicate that in order for expression of the *yolk proteins* in the female fat body to be maximal, a positive regulation is required from the Dsx^F protein as well as the absence of the repressive Dsx^M protein. However, this extra increase in expression is small compared to the several hundred times increase seen between flies with and without Dsx^M, although it may still be physiologically relevant. While explanations for most of the above evidence can be made within a model which only allows for a repressive function for Dsx proteins, this direct evidence makes it much more likely that positive functions for Dsx proteins do exist.

Finally, a positive role for Dsx proteins is indicated by the observation that while certain abdominal neuroblasts undergo extra divisions in the male, no divisions are seen in either sex when the flies carry *dsx* null mutations (Taylor & Truman, 1992). Conversely, both *XX* and *XY* flies carrying a *tra* null allele exhibit the extra divisions. This suggests that Dsx^M positively causes these neuroblasts to enter the 'extra-divisions' pathway while Dsx^F positively causes the same cells to enter the 'no-extra-divisions' pathway.

The studies on the *yp*'s described above indicate that Dsx^M and Dsx^F proteins exert opposite influences upon the genes while binding to exactly the same sites. How can this occur? These disparate functions must result from the regions of these proteins which are not common. It is likely that the differential effects are brought about by repressive and activatory functions, mediated by the non-common region of the Dsx proteins, upon other transcriptional activators which bind to promoters, in the same way as AEF-1, C/EBP and BBF-2 bind to the *yp* FBE. In this way, the absence of either Dsx protein allows a basal rate of transcription from sex-specifically expressed genes under the control of non sex-specific activator proteins. The binding of the

appropriate Dsx protein would then boost the expression to its maximal level, while binding of the inappropriate Dsx protein would inactivate the gene.

Finally, a role for the Intersex protein can be proposed with reference to the binding of a yeast protein; the MATα2 homeodomain protein. This protein binds to its cognate site as a dimer with the aid of the MCM1 protein as shown in figure 1.16 (Wolberger *et al.*, 1991). The genetic evidence discussed above shows that Ix is required for Dsx function. One model to account for this would be that Ix protein is required for high efficiency binding of Dsx proteins to their cognate binding sites in sex differentiation genes. Elucidation of this model awaits cloning of the *ix* gene.

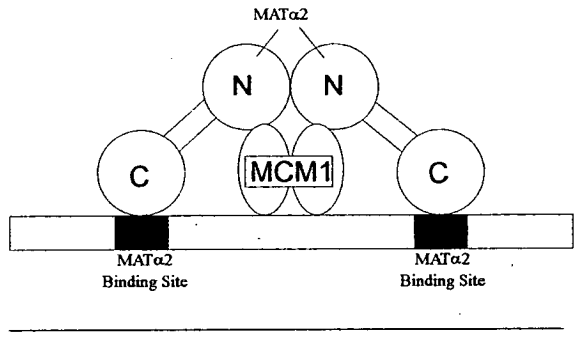


Figure 1.16
Diagram showing mode of action of cooperative binding of the yeast MCM1 and MATα2 DNA-binding proteins.

1.4.3 BRANCHES IN THE HIERARCHY ABOVE *DSX*

It is apparent that not all sexual functions are controlled directly by *dsx*. The overall size of the fly is affected by *Sxl* mutations but not by mutations in genes epistatic to *Sxl* in the hierarchy. Thus, *tra* or *tra-2* pseudomales are much larger than their XY counterparts. *tra-2* is also required for processes where *dsx* is not. This includes the production of a somatic feminising signal to developing pole cells (see earlier discussion of germline sex determination) and the specification of the male-specific muscle of Lawrence (MOL) in the fifth abdominal segment. Transplantation of

myoblasts between male and female have shown that the identity of the MOL is not autonomous but is more likely to depend upon the sex of the innervating axons (Lawrence & Johnston, 1986). It is believed that *tra* and *tra-2* have a role to play in this innervation, since *tra* or *tra-2* mutations can allow the development of the muscle in chromosomal females while *dsx* mutations do not have this effect (Taylor, 1992).

There is also some evidence that there may be a branch of regulatory genes, leaving the hierarchy at *tra/tra-2*, which represses aspects of male courtship behaviour (Belote & Baker, 1987; Gailey *et al.*, 1991; Taylor *et al.*, 1994). Male courtship consists of tapping the female with forelegs, orienting towards and following her, vibrating a wing to produce courtship 'song', licking the female genitalia and copulation (Bastock & Manning, 1955). Initially, use of a *tra-2* temperature sensitive allele showed that female repression of certain elements of male courtship behaviour are dependent upon *tra-2* (Belote & Baker, 1987). It was later observed that *XX* flies carrying either a *dsx* null allele or the dsx^D allele, which constitutively produces the male Dsx^M protein, did not exhibit any aspects of male courtship behaviour (Taylor *et al.*, 1994). This indicates that neither Dsx^M nor Dsx^F play a part in male courtship regulation. However, the possibility of a separate *dsx*-independent pathway which regulates male courtship is a contentious issue at present. This is due to the observation that *XY/dsx* flies court much less frequently than wild type flies, elicit higher levels of courtship, and have a disrupted courtship song (McRobert & Tompkins, 1985; Villella & Hall, 1996). Taylor *et al.*, 1994, suggested that the CNS of *XY/dsx* flies could still be essentially male, with lower levels of courtship and higher levels of elicitation of courtship being due to generalised developmental defects caused by inappropriate expression of genes usually repressed by the Dsx proteins. Since young wild type males exhibit lower levels of courtship and higher levels of elicitation of courtship than adults, a retardation of development might cause these characteristics to be prolonged. In support of this theory, the courtship song of *XY/dsx* flies, although perturbed, was not nearly as discomposed as the song of gynandromorphic flies in which much of the thoracic ganglion has the *XX*

genotype (Taylor *et al.*, 1994). This suggests that the CNS of *XY/dsx* flies may still be genetically male. In addition, *XY/dsx* flies are male-like in the nature of the pheromones which they produce (Jallon *et al.*, 1988). However, recently more in-depth studies on the courtship behaviour of *XY/dsx* flies by Villella & Hall, 1996, have suggested that the elicitation of courtship by these mutants is not likely to be due to maturational factors. However, these same authors report that the courtship song of *XY/dsx* flies was "quite similar" to that of wild-type males. In general, the analysis of courtship behaviour is complicated by variations of particular behaviour seen, dependent upon which *dsx* allele is used and what methods are used to score the behaviour. For example, in two separate studies on *XY/dsx* null mutants, both using the dsx^1 allele, McRobert & Tompkins, 1985, reported elicitation of courtship by the mutants, while Jallon *et al.*, 1988, reported no such effect. All of the above evidence is open to different interpretations. On balance, however, the evidence suggests that some aspects of male courtship behaviour are regulated by genes downstream of *dsx*, while other aspects are dependent upon *tra/tra-2* and not upon *dsx*.

If there is a *dsx*-independent *tra/tra-2*-mediated regulatory pathway which represses certain aspects of male courtship behaviour, it may be that this pathway has shared components with the *tra/tra-2*-mediated pathway which controls repression of the muscle of Lawrence. Interestingly, certain alleles of the gene *fruitless* (*fru*) result in loss of the MOL and in non-specific courting behaviour, such that *XY/fru* flies court males just as much as females (Gailey *et al.*, 1991; Taylor *et al.*, 1994). Wild-type males usually only court adult females and very young males. It may be that the *fru* gene lies in the *tra/tra-2*-mediated pathways which regulate male courtship behaviour and MOL development. In support of such a model, ectopic expression of the female form of *tra* in the antennal lobes or mushroom bodies of the male brain causes similar non-specific courting behaviour as with some *fru* alleles (O'Dell *et al.*, 1995; Ferveur *et al.*, 1995). However, it has recently been observed that *XY/dsx* mutants can also display non-specific courting behaviour (Villella & Hall, 1996). Thus, it seems likely that this particular behaviour is not under the exclusive control

of a *dsx*-independent pathway. However, it may be that non-specific courting is a 'default' state which results from general perturbation of the genetic control of courtship behaviour. If this is the case, mutation of a number of courtship-determining genes may result in this phenotype even if they are not all part of a common pathway.

1.4.4 CONTINUAL DEPENDENCE UPON THE HIERARCHY

Analysis of *yp* gene expression in flies carrying a temperature sensitive *tra-2* allele shows that *yp* expression remains under continual hierarchy control in the fat body, but not in the follicle cells of the ovary (Bownes *et al.*, 1990). Regulation of the ovary-specific expression pattern of the genes which go to make up the chorion and vitelline membrane of the oocyte is independent of the genes of the sex determination hierarchy (Waring & Mahowald, 1979; Fargnoli & Waring, 1982; Kafatos *et al.*, 1985). This suggests that the sex determination hierarchy genes act to continually regulate genes with non-gonadal sex-specific expression patterns, but are not required to maintain or repress expression of genes with gonad-specific expression patterns. Thus, while the sex determination hierarchy genes are required to determine the identity of the gonad, the maintenance of sex-specific expression patterns within the tissue appears to be under the control of tissue-specific factors. A number of studies support this hypothesis.

Two accessory gland-specific proteins are encoded by the *msP355a* and *msP355b* genes (Monsma & Wolfner, 1988; Monsma *et al.*, 1990). The *msP355a* gene encodes a protein with similarity to a hormone precursor and the *msP355b* gene encodes a small acidic protein. Both proteins are transferred to the female during copulation. Transcripts from these genes are detected in accessory gland tissue of *XX/tra-2*^{ts} pseudomales raised at the restrictive temperature. When these flies are shifted to the permissive temperature, the transcripts remain. Thus, in the same way as with the gonadal expression of the *yp* genes, the *msP355a* and *msP355b* genes appear to

require hierarchy genes in order to determine the tissue in which they are expressed but do not exhibit continual dependence.

The screen performed by Schäfer, 1986a, in which the *Mst87F* gene was isolated (section 1.1.3.2.3), also uncovered four other genes with accessory gland-specific expression patterns. Northern analysis of one of these accessory gland-specific transcripts, using a *tra-2* mutant, shows that determination of accessory gland tissue must occur for this transcript to be produced but does not indicate whether any continual regulation by *tra-2* is occurring (Schäfer, 1986b).

A second differential screen performed by DiBenedetto *et al.*, 1987, led to the isolation of one ovary-specific transcript, five testis-specific transcripts and one accessory gland-specific transcript. The accessory gland-specific transcript was shown not to be under the continual control of *tra-2*.

In XX gynandromorphs, the study of XO cell clones in different regions of the nervous system has enabled the identification of regions which are important for the production of certain male characteristics (Hodgkin, 1991). Correct male courtship requires a male brain and the fidelity of the male song depends upon the thoracic ganglion being chromosomally male. The continuing control of these tissues by at least one gene of the sex determination hierarchy is shown by adult female temperature shift experiments using the *tra-2*^{ts} allele. *XX*; *tra-2*^{ts} adults can exhibit aspects of male behaviour when shifted to the restrictive temperature. This means that at least some aspects of the dimorphic nervous system are continuously under the control of *tra-2*.

The sexual phenotype the genitalia and analia is also constantly dependant upon the expression of the *tra-2*^{ts} allele. This is shown by temperature shift experiments which shift the sex of the analia between the male and female fates (Belote & Baker, 1982; Epper & Bryant, 1983).

One system which does not fit in with the above model is the sex-specific pattern of abdominal neuroblast divisions discussed earlier (Taylor & Truman, 1992). Once these cells have entered either the male-specific or female-specific division pathway, temperature-shifts using hierarchy gene temperature sensitive alleles have no effect.

The *glucose dehydrogenase* (*Gld*) gene of *Drosophila* is expressed in very specific patterns in the somatic component of both male and female gonads (Feng *et al.*, 1991). Unlike the gonad-specific transcripts discussed above, the expression pattern of *Gld* can be altered by hierarchy gene mutants (including *dsx*) in adults.

Thus, it seems that not all genes will fit the paradigm of the *yp* genes. However, the body of evidence suggests that the majority of genes with somatic gonad-specific expression patterns will be hierarchy gene-independent, having come under the control of tissue-specific factors, following hierarchy gene-dependent determination of gonad cells.

1.4.5 OTHER GENES WITH SEX-SPECIFIC EXPRESSION PATTERNS

Following copulation, ovulation and oviposition is stimulated in the female. Females also indicate unreceptivity, by extending their ovipositor towards any courting males. These responses can be induced by the injection of male accessory gland secretions into virgin female abdomens (Leahy & Lowe, 1967). The 36 amino acid sex peptide is synthesised in the accessory gland and is capable of eliciting the oviposition and rejection responses in a purified form (Chen *et al.*, 1988).

The *janus* region contains two overlapping transcription units *janA* and *janB* (Yanicostas *et al.*, 1989). The *janA* unit produces a non sex-specific transcript and two male-specific transcripts. The *janB* unit produces a single male-specific transcript. All of the male-specific transcripts appear to be germline-specific.

The *Andropin* gene encodes an antibacterial protein which is localised to the male ejaculatory duct (Samakovlis *et al.*, 1991). The regulation of this gene has not yet been examined.

A differential screen performed using cDNA prepared from pre-blastoderm and gastrula embryos uncovered the *yema* locus which encodes four nurse cell-specific transcripts (Ait-Ahmed *et al.*, 1987). Again, little is known about the regulation of this locus.

The *Dromsopa* gene produces a male-specific transcript encoding a protein which contains glutamine-rich OPA repeats (Grabowski *et al.*, 1991). It is not known precisely which tissue this transcript is localised to, or whether it is regulated by the sex determination hierarchy genes.

To date, no genes have been isolated which exhibit non-gonadal sex-specific expression patterns other than the *yp* genes and the genes of the sex determination hierarchy. Genes which would be involved in differentiation of non-gonadal sex-specific features would be expected to express non-gonadal sex-specific transcripts. Therefore, the elucidation of non-gonadal sex differentiation awaits the cloning of genes which express non-gonadal sex-specific transcripts. It is likely that such genes have not been isolated so far via differential screens because their transcripts are present at lower levels than abundant gonad-specific transcripts.

The following chapters describe the cloning and characterisation of a gene expressing non-gonadal sex-specific transcripts isolated via a differential screen designed to isolate candidate non-gonadal sex differentiation genes.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 SOLUTIONS

Chemicals were obtained from SIGMA, BDH and Aldrich. Modification enzymes, such as restriction enzymes, Taq polymerase, nucleic acid ligases and phosphatases were obtained from Pharmacia, Boehringer Mannheim, New England Biolabs (NEB), GIBCO BRL, USB and NBL. Radioactive isotopes were obtained from Amersham and ICN Pharmaceuticals.

Solutions were prepared in sterile double-distilled water, unless otherwise stated, and sterilised by autoclaving at 15 psi for 15 minutes.

The composition of solutions not described in the main text is given below;

SOLUTION	**COMPOSITION**
TE	1mM EDTA (pH8.0), 10mM Tris-HCl (pH7.5).
0.5M EDTA	0.5M Diaminoethanetetra-acetic acid (pH8.0)
10X Agarose-gel loading buffer	100mM EDTA (pH8.0), 0.1% (w/v) Bromophenol Blue, 0.1% Xylene Cyanol FF, 30% (v/v) Glycerol.
2X Protein gel loading buffer	100mM Tris-HCl (pH 6.8), 200mM DTT, 4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue, 20% (v/v) Glycerol.
10X MOPS	0.2M Sodium-MOPS (pH7.0), 50mM Sodium Acetate, 10mM EDTA.
10X TBE	0.89M Tris-Borate, 0.89M Boric acid, 10mM EDTA.
20X SSC	3M Sodium Chloride, 0.3M Tri-Sodium Citrate (pH7.0).
20X SSPE	3.6M Sodium Chloride, 20mM Sodium Hydrogen Phosphate (pH 7.4), 20mM EDTA (pH 8.0).
10X PBS	0.1M Sodium Phosphate (pH 7.5), 1.3M Sodium Chloride.

Ringer's solution	1 litre: 3.2g Sodium Chloride, 3.0g Potassium Chloride, 1.8g Magnesium Sulphate, 0.69g Calcium Chloride, 1.79g Tricine, 3.6g Glucose, 17.1g Sucrose.
100X Denhardts Solution	2% (w/v) Bovine Serum Albumin, 2% (w/v) Polyvinylpyrrolidone, 2% (w/v) Ficoll.
Samlon Sperm DNA	10mg/ml, sonicated and denatured.
OLB Solution O	0.125M Magnesium Chloride, 1.25M Tris-HCl (pH8.0).
OLB Solution A	0.95ml Solution O, 18ul β-Mercaptoethanol, 25ul 20mM dATP, 25ul 20mM dTTP, 25ul 20mM dGTP.
OLB Solution B	2M HEPES (pH6.0).
OLB Solution C	Hexadeoxyribonucleotides @ OD=90 units/ml (Sigma).
OLB	Mixture of OLB Solutions A, B and C, in the ratio 2:5:3, respectively.
FSB	50% (v/v) Formamide, 25% (v/v) Formaldehyde (@ 14.8% [w/v]), 25% (v/v) 10X MOPS.
NES	50% (v/v) FSB, 7.5% (v/v) 10X Agarose gel loading buffer, 42.5% (v/v) DEPC-treated dH_2O.
DNA extraction buffer	50mM Tris-HCl (pH9.0), 0.1M EDTA (pH 8.0), 0.2M Sodium Chloride, 1mg/ml Ribonuclease A.
RNA extraction buffer	7M Urea, 350mM Sodium Chloride, 100mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0), 2% (w/v) SDS.
mRNA lysis/binding buffer	100mM Tris-HCl (pH 8.0), 500mM Lithium Chloride, 10mM EDTA (pH 8.0), 1% (w/v) Lithium Dodecyl Sulphate, 5mM DTT.
mRNA washing buffer	10mM Tris-HCl (pH 8.0), 0.15M Lithium Chloride, 1mM EDTA (pH 8.0), 0.1% (w/v) Lithium Dodecyl Sulphate.
mRNA elution solution	2mM EDTA (pH 8.0).
Magnetic bead recon. solution	0.1M Sodium Hydroxide.
Magnetic bead storage buffer	250mM Tris-HCl (pH 8.0), 20mM EDTA (pH 8.0), 0.1% (v/v) Tween-20, 0.02% Sodium Azide.

Miniprep solution I	50mM Glucose, 10mM EDTA (pH 8.0), 25mM Tris-HCl (pH 8.0).
Miniprep solution II	0.2M Sodium Hydroxide, 1% (w/v) SDS.
Miniprep solution III	3M Potassium Acetate, 11.5 % (v/v) Acetic Acid.
Southern Denaturing solution	1.5M Sodium Chloride, 0.5M Sodium Hydroxide.
Southern Neutralising solution	1.5M Sodium Chloride, 0.5M Tris-HCl (pH 7.2), 1mM EDTA (pH 7.7).
APS	25% (w/v) Ammonium Persulphate.
Acid Phenol	Phenol (pH 4.3) was obtained from Sigma, pre-equilibrated with 0.1M Citrate buffer.
Phenol	Phenol (pH 8.0) was obtained from Sigma, pre-equilibrated with 0.1M Tris-HCl (pH 8.0).
Phenol/Chloroform	Phenol (pH 8.0) was mixed with Chloroform and iso-amyl alcohol in the ratio 25:24:1, respectively.
10mM dNTPs	10mM each of dATP, dTTP, dGTP and dCTP.
10X PCR buffer	200mM Tris-HCl (pH 8.4), 500mM Potassium Chloride.
5X 1ststrand cDNA synthesis buffer	250mM Tris-HCl (pH 8.3), 375mM Potassium Chloride, 15mM Magnesium Chloride.
5X T4 DNA Ligase buffer	250mM Tris-HCl (pH 7.6), 50mM Magnesium Chloride, 5mM ATP, 5mM DTT, 25% (w/v) Polyethylene glycol (8000).
5X TdT buffer	0.5M Potassium Cacodylate (pH 7.2), 10mM Cobalt Chloride, 1mM DTT.
10X Transcription buffer	400mM Tris-HCl (pH 8.0), 60mM Magnesium Chloride, 20mM Spermidine, 100mM Sodium Chloride, 100mM DTT, 1unit/ul RNAase inhibitor.
DNAase I	20mg/ml Deoxyribonuclease I in 50% (v/v) glycerol.
RNAaseA	20mg/ml Ribonuclease A in 50% (v/v) glycerol. DNAase contamination was removed by boiling and centrifugation.
Proteinase K	20 mg/ml in 50% (v/v) glycerol.
Lysosyme	8 mg/ml in dH_2O.
10X SAP buffer	200mM Tris-HCl (pH 8.8), 100mM Magnesium Chloride.

SAP dilution buffer	50mM Tris-HCl (pH 8.0).
Ethidium Bromide	10mg/ml in dH_2O.
'Phage storage buffer (SM)	100mM Sodium Chloride, 8.1mM Magnesium Sulphate, 50mM Tris-HCl (pH 7.5), 0.5% (v/v) Gelatin.
'Phage adsorption buffer	10mM Calcium Chloride, 10mM Magnesium Chloride.
X-Gal	20mg/ml 5-bromo-4-chloro-3-indoyl-β-galactoside in dimethyl formamide.
IPTG	1M Isopropyl-β-D-thiogalactoside.
Ampicillin	50mg/ml in dH_2O.
Kanamycin	10mg/ml in dH_2O.
Tetracycline	5mg/ml in Ethanol.
Carbenicillin	50mg/ml in dH_2O.
Chloramphenicol	34mg/ml in dH_2O.
Leupeptins	10mg/ml in dH2O
Pepstatin A	1mg/ml in Ethanol
PMSF	17.4mg/ml in Isopropanol
Lumigen Buffer 1	100mM Maleic Acid, 150mM Sodium Chloride, pH 7.5.
Lumigen Buffer 2	1% (w/v) Blocking Reagent (Boehringer Mannheim) in Buffer 1.
Lumigen Buffer 3	100mM Tris-HCl (pH 9.5), 100mM Sodium Chloride, 50mM Magnesium Chloride.
Lumigen Washing Buffer	0.3% (v/v) Tween-20 in Buffer 1.
DIG DNA labelling mix	1mM dATP, 1mM dCTP, 1mMdGTP, 0.65mM dTTP, 0.35 mM DIG-dUTP, pH 7.5.
Qiagen L1	20mg/ml RNAaseA, 6mg/ml DNAaseI, 0.2mg/ml BSA, 10mM EDTA (pH 8.0), 100mM Tris-HCl (pH 7.5), 300mM Sodium Chloride.
Qiagen L2	30% (w/v) Polyethylene Glycol (6000), 3M Sodium Chloride.
Qiagen L3	100mM Tris-HCl (pH 7.5), 100mM Sodium Chloride, 25mM EDTA (pH 8.0).
Qiagen L4	4% (w/v) SDS.
Qiagen L5	2.55M Potassium Acetate (pH 4.8).

Qiagen P1	50mM Tris-HCl (pH 8.0), 10mM EDTA (pH8.0), 0.1mg/ml RNAaseA.
Qiagen P2	200mM Sodium Hydroxide, 1% (w/v) SDS.
Qiagen P3	3M Potassium Acetate (pH 5.5).
Qiagen QBT	750mM Sodium Chloride, 50mM MOPS, 15% (v/v) Ethanol, 0.15% (v/v) Triton X-100, pH 7.0.
Qiagen QC	1M Sodium Chloride, 50mM MOPS, 15% (v/v) Ethanol, pH 7.0.
Qiagen QF	1.25M Sodium Chloride, 50mM Tris, 15% (v/v) Ethanol, pH 8.5.
Qiaex QX1	3M Sodium Iodide, 4M Sodium Perchlorate, 50mM Tris-HCl (pH 7.5), 0.1% (w/v) Sodium Sulphite.
Qiaex QX2	8M Sodium Perchlorate.
Qiaex QX3	70% (v/v) Ethanol, 100mM Sodium Chloride, 10mM Tris, 1mM EDTA, pH 7.5.
Boehringer restriction buffer A	33mM Tris-HCl, 10mM Magnesium Acetate, 66mM Potassium Acetate, 0.5mM DTT, pH 7.5.
Boehringer restriction buffer B	10mM Tris-HCl, 5mM Magnesium Chloride, 100mM Sodium Chloride, 1mM β-Mercaptoethanol, pH 7.5.
Boehringer restriction buffer L	10mM Tris-HCl, 5mM Magnesium Chloride, 1mM Dithioerythritol, pH 7.5.
Boehringer restriction buffer M	10mM Tris-HCl,10mM Magnesium Chloride, 50mM Sodium Chloride 1mM Dithioerythritol, pH 7.5.
Boehringer restriction buffer H	50mM Tris-HCl, 10mM Magnesium Chloride, 100mM Sodium Chloride, pH 7.5.
L-Broth	1 litre: 10g Difco Bacto Tryptone, 5g Difco Bacto Yeast Extract, 5g Sodium Chloride.
L-Agar	1 litre: 16g Difco Bacto Tryptone, 10g Difco Bacto Yeast Extract, 5g Sodium Chloride.
Top Agarose	0.7% Agarose in L-Broth.
SOC	L-Broth supplemented with 2.5mM Potassium Chloride, 10mM Magnesium Chloride, 10mM Magnesium Sulphate, 20mM Glucose.

2.1.2 BACTERIOPHAGE LIBRARIES.

2.1.2.1 Stratagene *Drosophila* Genomic DNA library.

This library was purchased from Stratagene and utilises the Stratagene Fix^{TM}II bacteriophage lambda (λ) vector. The vector contains two adjacent *Xho*I sites in its multiple cloning site, which is flanked by convergent T3 and T7 transcriptional promoters. The vector was digested with *Xho*I and the resulting sticky ends were partially filled to create Sau3A-compatible ends. Vector was then added to a Sau3A digest of *Drosophila* genomic DNA and ligated. The vector is capable of accepting insert sizes of 9-23kb.

2.1.2.2 Adult Body cDNA Library.

This library was obtained from Prof. M. Ashburner (University of Cambridge), and was constructed in the bacteriophage lambda ('phage λ) vector NM1149. RNA was extracted from *Drosophila* male and female carcasses (lacking gonads) and double-stranded cDNA synthesised. Internal restriction sites were protected by methylation and a linker sequence was ligated to both ends of the cDNA, which introduced an *Eco*RI site at the 5' end and a *Hin*dIII site and an *Eco*RI site at the 3' end (Dorssers & Postmes, 1987). In this way, *Eco*RI/*Hin*dIII double-digestion of the cDNA produced cDNAs with a *Eco*RI-compatible site at the 5' end and a *Hin*dIII-compatible site at the 3' end. The cDNA's were then cloned into the *Eco*RI and *Hin*dIII sites of the NM1149 vector.

2.1.3 PLASMID VECTORS AND BACTERIAL HOSTS

Details of all plasmid vectors and bacterial hosts used are given below;

PLASMID	RELEVANT GENOTYPE	COMMENTS
pBluescript	*amp*^{r}	Phagemid used for general cloning procedures and generating single-stranded DNA templates for DNA sequencing. Contains multiple cloning site inserted, in-frame, into β-galactosidase (LacZ) gene. The LacZ gene is stimulated by IPTG to produce β-galactosidase which catalyses the breakdown of X-Gal, creating a blue reaction product. Thus, blue colonies are produced, unless insert DNA is present in the multiple cloning site.
pGEX^{3X}	*amp*^{r}	Glutathione-S-Transferase (GST) Fusion Protein expression vector, containing a multiple cloning site downstream of GST sequence and an IPTG-inducible Ptac promoter.

HOST	RELEVANT GENOTYPE	COMMENTS
XL-1 Blue	*Rec A1*, *end A1*, *gyr A96*, *thi*, *hsdR17*, *supE44*, *rel A1*, λ-, Δ(*lac*), [F', *proAB*, *lacI*^{q}, *ZΔM15*, Tn10(*tet*^{r})]	Used to propagate pBluescript clones. Tet selection is required to maintain F plasmid which encodes factors required for blue/white colour selection and single-stranded DNA preparation from pBluescript phagemid.
C600	F$^-$, *thi-1*, *leub6*, *lacY*^{1}, *tonA21*, *supE44*.	Used to propagate both 'phage λ libraries described above.
BL21	DE3 LysS	Used as host for pGEX expression vectors. Chloramphenicol selection is required to maintain LysS plasmid, encoding factors preventing expression from Ptac promoter in absence of IPTG and enable efficient lysis of cells via freeze-thawing.

2.1.4 *DROSOPHILA* STRAINS

Drosophila stocks were maintained at 18°C or 25°C on cornmeal food (1 litre: 25g cornflour, 50g sugar, 17.5g yeast, 10g agar, boiled, cooled to 40°C and poured into bottles or vials to set). Nipagen fungicide was added to 4.5ʋg/l. Antibiotics were added only on an occasional basis to prevent selection for resistant bacterial strains. In the event of mite infestations, filter paper strips, soaked in 3% (v/v) Benzyl Benzoate in ethanol and allowed to air dry, were placed on the cooled food prior to introduction of any flies. The relevant details of *Drosophila* strains used in the following analysis are shown below. All mutant *Drosophila* stocks were obtained from Prof. R. Nöthiger (Zoologisches Institut der Universität, Zürich).

STOCK	GENOTYPE	COMMENTS
Oregon R (OrR)	Wild Type.	
2-10	B^sY; $tra2^{ts1}$ *bw* /In(2LR)*CyO*	*XY* flies have bar eyes. Permissive temperature, 16°C. Restrictive temperature, 25°C. $tra2^{ts1}$ heterozygotes have curly wings. $tra2^{ts1}$ homozygotes have straight wings and brown eyes.
2-11	B^sY; *tra-*2^{ts2} *bw* / *CyO*	As 2-10, but the *tra-*2^{ts2} allele is less severe than $tra2^{ts1}$, showing increased viability.
O3-10	B^sY; *dsx* p^p / TM6; *ubx*	*XY* flies have bar eyes. *dsx* null homozygotes have pink eyes. The TM6 balancer is homozygous lethal.
O13-1	B^sY; *dsx* p^p / TM6	As O3-10, but a different *dsx* null allele.
tra^{FEM}	B^sY; Df(3L)82j7 *Ki roe* p^p hs[tra^{FEM}] / dsx^D *Sb e*	*XY* flies have bar eyes. Heterozygotes exhibit kinked, stubbled bristles. dsx^D homozygotes have stubbled bristles and ebony body colour. tra^{FEM} homozygotes have kinked bristles and roughened, pink eyes.

Both *dsx* and $tra2^{ts}$ homozygotes exhibit poor viability and trans-heterozygotes (e.g. $tra2^{ts1}$ / $tra2^{ts2}$) were constructed to take advantage of hybrid vigour.

2.2 METHODS

2.2.1 GENERAL METHODS

2.2.1.1 Phenol extraction.

Nucleic acid solutions were deproteinised by vortexing with an equal volume of Tris-HCl-equilibrated phenol (pH 8.0). The aqueous and organic phases were separated by centrifugation in an eppendorf microcentrifuge (12Krpm for 1min) and the upper (aqueous) phase removed to a fresh tube. The nucleic acid solution was then re-extracted with an equal volume of phenol/chloroform and again with an equal volume of chloroform/iso-amyl alcohol (24:1) to remove any traces of phenol.

2.2.1.2 Butanol extraction.

Ethidium Bromide was removed from nucleic acid solutions by vortexing with an equal volume of dH_2O-saturated butanol. The aqueous and organic phases were separated by centrifugation in an eppendorf microcentrifuge (12Krpm for 1min) and the lower (aqueous) phase removed to a fresh tube. This was repeated until no traces of Ethidium Bromide remained in the aqueous phase.

2.2.1.3 Precipitation of nucleic acids.

Nucleic acids were precipitated by addition of 0.1X volumes of 3M Sodium Acetate (pH 4.8) and 2.5X volumes of either ethanol or isopropanol. The solution was mixed and incubated on ice for 15min. Nucleic acids were pelleted by centrifugation at 4°C in an Eppendorf microcentrifuge at 17Krpm for 15min. The supernatant was removed and the pellet washed in 80% (v/v) ethanol. The pellet was then dried *in vacuo* and resuspended in TE or dH_2O.

2.2.1.4 Quantitation of nucleic acid solutions.

The OD_{260} of the nucleic acid solution was determined in a suitable spectrophotometer. DNA solutions of 0.05 mg/ml, and RNA solutions of 0.04 mg/ml, have an OD_{260} of 1.0. Thus, the concentration of any nucleic acid solution, of known OD_{260}, can be calculated. Dilutions of nucleic acid solutions were used which produced OD_{260} readings within the range 0.2-1.0, since readings outwith this range are unreliable. Concentrations of nucleic acid solutions were also determined by comparison with samples of known concentration on Ethidium Bromide-stained agarose gels. A protein-free nucleic acid solution is indicated by an OD_{260}/OD_{280} reading in the range 1.8-2.0, with readings significantly lower than this indicating protein contamination.

2.2.1.5 Agarose gel electrophoresis.

For analysis of DNA samples, agarose was added to 1X TBE and dissolved by heating in a microwave oven. The gel mixture was allowed to cool to approximately 60°C and then poured into a gel forming apparatus. 0.1X vols of 10X agarose gel loading buffer was added to DNA samples and the samples were loaded into the wells, under 1X TBE, in the gel apparatus. Gels were run in 1X TBE at the voltages recommended by the apparatus manufacturer. DNA fragments were visualised either by addition of Ethidium Bromide (to 0.5υg/ml) to the gel mix prior to pouring, or by staining the gel after running, in 1X TBE containing 0.5υg/ml Ethidium Bromide. Gels were destained in 1X TBE, to remove excess Ethidium Bromide. For most applications, 1.0% (w/v) agarose gels were used. Lower agarose concentrations were used where the resolution of DNA fragments larger than around 2.0kb was required (e.g. 0.8% for 2-10kb fragments). Higher agarose concentrations were used to resolve small DNA fragments (e.g 1.2% for 0.2-1.0kb fragments). The molecular weights of DNA molecules were determined with reference to Gibco BRL '1kb DNA ladder' molecular weight markers.

For analysis of RNA samples, denaturing agarose gels were used. Agarose was added to RNAase-free dH_2O and dissolved by heating in a microwave oven. The gel mixture was allowed to cool to approximately 60°C and 10X MOPS added to a final concentration of 1X. Formaldehyde was then added to a final concentration of 2.2M. The mixture was then poured into gel forming apparatus and allowed to set. RNA samples were denatured by adding an equal volume of FSB and heating to 65°C for 15 minutes followed by snap-cooling on dry ice. Where visualisation of the samples was required, Ethidium Bromide was added to the samples to a final concentration of 0.01υg/υl. The samples were loaded and the gel run in 1X MOPS buffer, according to the voltages recommended by the gel apparatus manufacturer. The molecular weights of RNA molecules were calculated with reference to transcripts of known size (e.g. 18s small subunit ribosomal protein transcript, ribosomal protein 49 [rp49] transcript, ribosomal RNA transcripts, α-tubulin transcript and *dsx* transcripts).

2.2.1.6 DEAE purification of DNA fragments from agarose gels.

DNA was separated on 1X TBE agarose gels. A slit was cut in front of the required band to enable insertion of DEAE membrane (Schleicher & Schnell). A piece of DEAE membrane was cut to the width of the slit and to a depth slightly greater than that of the slit. The membrane was activated by soaking for 5 minutes in 10mM EDTA, followed by soaking for 5 minutes in 0.5M Sodium Hydroxide. The membrane was then washed six times in excess dH_2O and stored in dH_2O at 4°C until required (for up to 3 weeks). The membrane was inserted into the slit and the gel was run until the band could be seen, under UV light, to be immobilised upon the membrane. The membrane, containing the DNA band, was then removed, rinsed in low salt buffer (150mM Sodium Chloride, 0.1mM EDTA), and placed into an Eppendorf tube. DNA was eluted from the membrane by incubating the membrane at 65°C in 300υl high salt buffer (1M Sodium Chloride, 0.1mM EDTA, 20mM Tris-HCl [pH 8.0]) for 30 minutes. The eluate was removed to a fresh tube and the

elution step repeated. Both eluates were combined, phenol/chloroform extracted and ethanol precipitated.

2.2.1.7 Qiaex purification of DNA fragments from agarose gels.

DNA was separated on 1X TBE agarose gels. The required band was excised from the gel in a minimum gel slice and placed in an Eppendorf tube. 300ul of QX1 solubilisation buffer and 30ul of 1M mannitol were added per 100mg of gel slice. 10ul of Qiaex DNA-affinity matrix was added and the gel slice was solubilised at 50°C for 10 minutes. Qiaex was briefly pelleted in a microcentrifuge, washed twice with 500ul of QX2 wash buffer and twice with 500ul of QX3 wash buffer. The pellet was dried *in vacuo* for 2 minutes. DNA was eluted in 20ul of TE at 50°C. A second elution step was carried out and the eluates were pooled.

2.2.1.8 Restriction endonuclease digestion of DNA.

In general, a 5-fold excess of restriction enzyme was used for digestion of DNA molecules (i.e. 5 units of enzyme per 1ug of DNA). Boehringer Mannheim restriction endonuclease reaction buffers were added to a final concentration of 1X. Restriction reactions were carried out at the recommended reaction temperature for the particular enzyme, for at least 1 hour.

2.2.1.9 Ligation of DNA molecules.

Where required, 5' phosphate groups were removed from the restriction endonuclease-digested ends using shrimp alkaline phosphatase, to prevent intramolecular religation. Generally, 0.1 units of shrimp alkaline phosphatase (SAP) was used per phosphatase reaction. SAP 10X reaction buffer (NEB) was added to a final concentration of 1X. Phosphatase reactions were carried out at 37°C for 60 mins, follwed by a 10 min incubation at 70°C to inactivate the enzyme.

Typically, vector and insert fragments were separated on agarose gels and purified via DEAE membrane. In general, a vector:insert ratio of 1:3 was used for standard ligation reactions, with 10-100ng of insert DNA being used. Ligations were carried out in 10υl reactions containing 1X ligase buffer and 0.01 units of T4 DNA ligase. Ligations were carried out overnight at 4°C. An aliquot of the ligation reaction was then transformed into *E. coli* competent cells.

2.2.1.10 Transformation of DNA into $CaCl_2$-competent *E. coli.*

Calcium Chloride competent cells were prepared as follows. 100ml of L-broth was inoculated with a single colony of an appropriate bacterial strain and incubated at 37°C, with shaking, until the bacterial cells were in Log growth phase (OD_{600} of 0.5-1.0). The cells were harvested by centrifugation (2 Krpm, 10 mins @ 4°C) and resuspended in an equal volume of ice-cold 50mM Calcium Chloride. The cells were incubated on ice for 20 mins, re-pelleted, and resuspended in 0.1X volumes of ice-cold 50mM Calcium Chloride. The competent cells were then stored overnight on ice to maximise their efficiency. A volume of ligation reaction not greater then $1/10^{th}$ of the final volume was added to 100-200υl of competent cells in an Eppendorf tube. The cells/ligation mix was incubated on ice for 30 mins, heat-shocked at 42°C for 90 secs, and placed on ice for 1 min. 2XYT growth medium was added to a final volume of 1ml, and the mixture incubated at 37°C for 45 mins, to enable bacterial expression of newly-transformed antibiotic resistance genes. The transformed cells were pelleted by brief centrifugation in a microcentrifuge and resuspended in 100υl L-broth. The cells were then plated on petri dishes containing L-agar, supplemented with appropriate antibiotics. The plates were, covered, inverted and incubated overnight at 37°C.

2.2.1.11 Transformation of *E. coli* by electroporation.

Electroporation-competent cells were prepared as follows. 100ml L-Broth was inoculated with a single colony of an appropriate bacterial strain and incubated at

37°C, with shaking, until the cells were in Log growth phase (OD_{600} of 0.5-1.0). The cells were incubated on ice for 30 mins and pelleted by centrifugation at 4000xg for 15 mins at 4°C. The cells were resuspended in an equal volume of dH_2O, re-pelleted, and resuspended in 0.5X volumes of dH_2O. The cells were then pelleted, resuspended in 2ml 10% (v/v) glycerol, re-pelleted, and resuspended in 200υl 10% (v/v) glycerol. Ligation reaction was precipitated and resuspended in dH_2O, to remove any ions which could result in short-circuiting of the electroporation apparatus. 1-2υl of ligation reaction was added to 40υl of competent cells, mixed, and placed on ice for 1 min, in an electroporation cuvette. A 5 msec pulse of 2.5 KVolts (25υF, 200Ω) was passed through the cells/ligation mix. 1ml of SOC medium was immediately added to the cuvette and mixed. The transformed cells were incubated for 45 mins at 37°C and plated as above.

2.2.1.12 Preparation of genomic DNA.

Approximately 5mg of *Drosophila* tissue was homogenised in a 1.5ml Eppendorf tube containing 50υl DNA extraction buffer. 50υl DNA extraction buffer, supplemented with 0.04% (w/v) SDS, was added. Proteinase K was added to a final concentration of 0.05 mg/ml and the homogenate incubated at 65°C for 60 mins. The mixture was then phenol extracted twice and nucleic acid was ethanol precipitated. The genomic DNA pellet was resuspended in TE.

2.2.1.13 Small scale preparation of plasmid DNA.

1.5ml of plasmid-transformed overnight bacterial culture was pelleted by brief centrifugation in a microcentrifuge. The cells were resuspended in 100υl of ice-cold miniprep solution I and incubated on ice for 5 mins. 200υl of freshly made miniprep solution II was added and mixed by inverting the tube 6-10 times. 150υl of ice-cold miniprep solution III was then added, mixed by inversion, and incubated on ice for 5 mins. Bacterial debris and genomic DNA were pelleted by centrifugation in a microcentrifuge at 17 Krpm for 15 mins (4°C). The supernatant was removed to a

fresh tube, phenol extracted, phenol/chloroform extracted and chloroform extracted. Plasmid DNA was ethanol precipitated, washed in 80% (v/v) ethanol, dried *in vacuo* and resuspended in TE. This method typically yielded 1-10ug of plasmid DNA.

2.2.1.14 Large scale preparation of plasmid DNA via Qiagen columns.

Tip-100 Qiagen columns were used to prepare up to 100ug of plasmid DNA. Essentially, this method is a scaled-up version of the Birnboim & Doly-type method described above. Qiagen DNA-affinity columns are used to enable purification of high quality, RNA-free plasmid DNA. Plasmid-transformed cells were harvested from 100ml of overnight culture by centrifugation at 6000xg for 15 min at 4°C. The cells were resuspended in 4 ml of ice-cold buffer P1. 4ml of buffer P2 was added, mixed by inversion, and the mixture incubated at room temperature for 5 mins. 4ml of ice-cold buffer P3 was then added, mixed by inversion, and the mixture incubated on ice for 15 mins. Bacterial debris and genomic DNA were removed by centrifugation at >20,000xg for 30 min at 4°C. The supernatant was centrifuged again at >20,000xg for 15 min at 4°C, to remove any traces of cellular debris. The supernatant was then applied to a qiagen Tip-100 (previously equilibrated with 4ml of QBT buffer) and allowed to fully enter the column. The column was washed with 20ml of buffer QC, and the plasmid eluted in 5ml of buffer QF. 0.7X volumes of isopropanol were added, mixed by inversion, and the precipitate pelleted by centrifugation at >15,000xg for 30 min at 4°C. The pellet was washed in 80% (v/v) ethanol, dried *in vacuo*, and resuspended in TE.

2.2.1.15 Large scale preparation of plasmid DNA via CsCl gradient.

The Caesium Chloride gradient method of plasmid preparation enables purification of several mg of extremely pure plasmid DNA. Plasmid-transformed cells were harvested from 250ml of overnight bacterial culture by centrifugation at 6000xg for 15 min at 4°C. The cells were resuspended in 5ml miniprep solution I and incubated on ice for 5 min. 10ml of freshly made miniprep solution II was added and mixed by

inverting the tube 6-10 times. 7.5ml of ice-cold miniprep solution III was then added, mixed by inversion, and incubated on ice for 5 mins. Bacterial debris and genomic DNA were pelleted by centrifugation at >20,000xg for 30 mins (4°C). The supernatant was removed to a fresh tube, containing 15ml Isopropanol, mixed, and incubated at room temperature for 15 min. Nucleic acid was pelleted by centrifugation at >15,000xg for 10 min at 4°C, washed in 80% (v/v) ethanol, dried *in vacuo* and resuspended in 3 ml TE. 50ul of 10mg/ml Ethidium Bromide was added to the 3 ml of plasmid solution, and the total density of the solution adjusted to 1.59 g/ml (+/- 0.01 g/ml) by addition of solid Caesium Chloride. The solution was placed in ultracentrifuge tubes and centrifuged at 45 Krpm for 20 hours at 4°C, in a swing-out rotor. The resulting plasmid band was visualised under UV light and extracted from the ultracentrifuge tube using an 18-gauge syringe needle. Ethidium Bromide was removed from the plasmid solution by butanol extraction. Plasmid DNA was ethanol precipitated, pelleted, washed in 80% (v/v) ethanol, dried *in vacuo* and resuspended in TE. Typically 1-2 mg of plasmid DNA were obtained using this method.

2.2.1.16 Preparation of total cellular RNA.

All solutions required for preparation of RNA were either rendered RNAase-free by treatment with Diethylpyrocarbonate (DEPC), or prepared from RNAase-free chemicals and RNAase-free dH_2O. DEPC was added to a final concentration of 10% (v/v), mixed thoroughly, and the mixture incubated at 37°C overnight. DEPC was then removed from the solution by autoclaving at 15 psi for 15 min. All glassware was rinsed with Chloroform and DEPC H_2O, and all plastics were treated with 0.1M Sodium Hydroxide and rinsed in DEPC H_2O. Where possible, all steps were carried out on ice, dry ice or in liquid Nitrogen, as appropriate.

Up to 100mg of *Drosophila* tissue was placed in a 1.5 ml Eppendorf tube and frozen in liquid Nitrogen. 400ul of RNA extraction buffer was added to the tissue and the tissue was homogenised using a hand-held homogeniser. The homogenate was

phenol/chloroform extracted with 200ul of acid phenol and 200ul of chloroform/iso-amyl alcohol (24:1). The homogenate was re-extracted with an equal volume of chloroform/iso-amyl alcohol (24:1) until no residue was observed between the aqueous and organic phases. 1ml of ethanol was added and the RNA precipitated at -70°C for at least 30min. RNA was pelleted by centrifugation in a microcentrifuge at 17Krpm for 15min at 4°C, washed in 80% (v/v) ethanol, dried *in vacuo* and resuspended in DEPC H_2O. RNA solutions were either used immediately, or frozen in liquid Nitrogen and stored at -70°C.

2.2.1.17 Preparation of PolyA$^+$ RNA.

Oligo $(dT)_{25}$ superparamagnetic polystyrene beads (Dynal) were used for preparation of PolyA$^+$ RNA. 250ul of bead suspension (5mg/ml) was removed from the main stock and placed in a fresh 1.5ml Eppendorf tube. The beads were magnetically captured, the storage buffer removed and the beads gently resuspended in 200ul lysis/binding buffer. Up to 50mg of *Drosophila* tissue was placed in a 1.5ml Eppendorf tube and frozen in liquid Nitrogen. 1ml of lysis/binding buffer was added to the tissue and the tissue was homogenised using a hand-held homogeniser. Debris was removed from the homogenate by centrifugation at 17Krpm for 5min at 4°C. The homogenate was then passed three times through a 21-gauge syringe needle to reduce viscosity caused by genomic DNA and fine cell debris. The 200ul aliquot of magnetic beads was placed in a magnet to capture the beads, and the supernatant removed. The 1ml homogenate was then added to the beads, gently mixed, and incubated for 8min at room temperature. The beads were washed three times with 1ml of washing buffer, with the beads being magnetically captured after each wash. PolyA$^+$ RNA was eluted from the beads in 20ul elution solution at 65°C for 2min. For Northern blot applications, PolyA$^+$ RNA was eluted in NES. The RNA was then denatured, supplemented with loading buffer, and loaded on a denaturing gel, as described above.

2.2.1.18 Southern blot analysis.

DNA samples were electrophoresed in an agarose gel, as described previously. The gel was soaked in 0.25M HCl until 10min after both Bromophenol Blue and Xylene Cyanol dyes had changed colour. The gel was rinsed in dH_20 and then soaked in denaturation buffer until 15min after loading buffer dyes had reverted to their original colours, or for 30min in total, whichever was longer. The gel was then rinsed in dH_2O and soaked in neutralisation buffer for 15min. A tray was filled with 20X SSC and a raised platform placed in the fluid. A wick, made from three sheets of 3M blotting paper, was placed on the platform, with the edges of the wick extending into the fluid. The gel was placed on the wick and a sheet of Hybond-N^+ nylon membrane layed on top of the gel. Three further sheets of 3M paper were placed on top of the Hybond-N^+. Finally, a stack of absorbent towels were layed on top and were weighted down by a 1kg weight. Transfer was allowed to continue for at least 16hrs. Before removing the Hybond-N^+ from the gel, the position of wells were marked on the membrane using a syringe needle. The membrane was then removed, rinsed in 2X SSC, dried at 65°C and the DNA fixed by UV cross-linking. The membrane was pre-hybridised in 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS, 0.1mg/ml salmon sperm DNA, 50% (v/v) formamide, overnight at 42°C. Denatured, labelled, probe was added and allowed to hybridise overnight at 42°C. Background hybridisation was removed by washing in 0.1X SSPE, 0.5% (w/v) SDS, at room temperature for 15min. If background signal persisted, further room temperature washes were carried out, followed by washes at increasing temperature. Generally, washing in 0.1X SSPE, 0.5% (w/v) SDS, at 65°C for 60min was sufficient to remove persistent background signal.

2.2.1.19 Northern blot analysis.

RNA samples were electrophoresed in an agarose-formaldehyde gel, as described above. RNA was transferred to Hybond-N membrane. As for Southern blotting, a capillary blot was set up using DEPC 20X SSPE as transfer buffer. No pre-treatment

of the gel was required. Transfer, fixation, pre-hybridisation, hybridisation and stringency washes were carried out as for Southern blotting.

2.2.1.20 Radiolabelling of DNA by random priming.

30-100ng of DNA, in a final volume of 32υl, was denatured by boiling for 3min and snap-cooled on dry ice for 1min. 10υl OLB, 1υl 20mg/ml BSA, 5υl [^{32}P]-dCTP (50υCi) and 2υl Klenow DNA polymerase (2 units) were added to the denatured DNA and mixed. The labelling reaction was carried out for 60min at 37°C. Unincorporated [^{32}P]-dCTP was removed by passing the labelling reaction through a Pharmacia gel-filtration 'Nick' column, according to the manufacturers instructions. Labelled probe was denatured at 95-100°C for 5min and snap-cooled on ice. The denatured, labelled probe could then be added to pre-hybridised Southern or Northern membranes.

2.2.1.21 Determination of radiolabelling efficiency.

1υl of radiolabelling reaction was diluted into 200υl dH_2O containing 10ug BSA. The mixture was cooled on ice for 10min, and tri-chloroacetic acid (TCA) added to 10% (w/v), to precipitate nucleic acid. Precipitate was collected by centrifugation and both pellet (representing incorporated label) and supernatant (representing un-incorporated label) were collected. The precipitated pellet was resuspended in 200υl dH_2O. Equal quantities of incorporated and un-incorporated label solutions were spotted onto separate Whatman GF/C glass filters and allowed to air dry. The filters were placed in scintillation vials, covered with scintillation fluid (6g/l butyl-PBD in toluene) and radioactivity quantitated in a scintillation spectrometer. Thus, the percentage incorporation could be calculated. Typically, 60-90% incorporation was achieved.

2.2.1.22 Autoradiography.

Detection of ^{32}P radiolabelled probe on Nylon membranes was performed using Cronex 4 Dupont X-ray film in light-tight cassettes at -70°C. Films were developed in an Agfa-1 automatic film processor. ^{35}S-labelled probes were detected using the same procedure, but were exposed to film at room temperature.

2.2.1.23 Labelling of DNA with digoxigenin-dUTP.

30-3000ng of DNA, in a final volume of 15υl, was denatured by boiling for 3min and snap-cooled on dry ice for 1min. 2υl hexanucleotide mix (Boehringer Mannheim), 2υl digoxigenin (DIG) labelling mix and 1υl Klenow DNA polymerase (1 unit) were added to the denatured DNA and mixed. The labelling reaction was carried out for 60min at 37°C. Unincorporated DIG-dUTP was removed by passing the labelling reaction through a Pharmacia gel-filtration 'Nick' column, according to the manufacturers instructions. Labelled probe was denatured at 95-100°C for 5min and snap-cooled on ice. The denatured, labelled probe could then be added to pre-hybridised Southern or Northern membranes.

2.2.1.24 Labelling of RNA with digoxigenin-UTP.

T7 transcriptional promoters present in pBluescript phagemid and FixII lambda vectors enable production of RNA molecules extending into insert sequences. These *in vitro* transcripts can be labelled with DIG-UTP. The following components were mixed in a 1.5ml Eppendorf tube; 14υl denatured DNA template (1υg), 2υl 10X Transcription buffer, 2υl DIG labelling mix, 2υl T7 RNA polymerase (20units/υl). The reaction was incubated at 37°C for 2-15min. Unicorporated DIG-UTP was removed as before, using Pharmacia 'Nick' gel-filtration columns. The labelled riboprobe was then denatured at 95-100°C for 5min, snap-cooled on dry ice, and added to pre-hybridised Northern or Southern membranes.

2.2.1.25 Detection of digoxigenin-labelled probes.

The chemiluminescent substrate Lumigen PPD (4-Methoxy-4-[3-phosphatephenyl] spiro[1,2-dioxetane-3,2'-adamantane; Boehringer Mannheim) was used to detect probes labelled with digoxigenin. Alkaline phosphatase catalyses the conversion of Lumigen PPD into an unstable intermediate which decomposes into light-emitting products. Thus, combination of Lumigen PPD with an anti-digoxigenin/alkaline phosphatase antibody conjugate (anti-DIG-AP) enables localised detection of digoxigenin via exposure to X-ray film. Following hybridisation of DIG-labelled probes to Southern or Northern filters, stringency washes were carried out as follows. The filter was washed 2 X 5min at room temperature with 2X SSC, 0.1% (w/v) SDS, 2 X 15min at 68°C with 0.1X SSC, 0.1% (w/v) SDS. The filter was then washed briefly in lumigen buffer 1 and blocked for 30min in lumigen buffer 2. The membrane was incubated for 30min in buffer 2 containing anti-DIG-AP at a final concentration of 75mU/ml. Excess antibody was removed by 2 X 15min washes in buffer 1 and the membrane was equilibrated for 5min in lumigen buffer 3. Lumigen PPD was added in buffer 3, at a final concentration of 0.1mg/ml and allowed to bathe the membrane for 10min. Excess fluid was blotted off with 3M paper and the membrane sealed in a plastic bag. The membrane was pre-incubated at 37°C for 15min and then exposed to X-ray film at room temperature for 15-25min. Films were developed as described above.

2.2.1.26 Determination of DIG-dUTP labelling efficiency.

Alkaline phosphatase catalyses the production of a coloured precipitate from the substrates X-phosphate (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt) and NBT (nitroblue tetrazolium salt). Thus, DIG-labelled probe can be directly visualised using anti-DIG-AP, and the quantity of labelled DNA can be directly compared to a control labelling reaction. Serial dilutions of an aliquot of the DIG labelling reaction (following removal of un-incorporated DIG-dUTP) were made. Equal quantities of each dilution were spotted onto Hybond N^+ membrane, allowed to air dry and fixed

by UV cross-linking. A control filter was also made up, using control DIG-labelled DNA provided by Boehringer Mannheim. The filters were washed firstly in lumigen buffer 1 for 1min, and then blocked in lumigen buffer 2 for 30min. The membranes were incubated for 30min in buffer 2 containing anti-DIG-AP at a final concentration of 75mU/ml. Excess antibody was removed by 2 X 15min washes in buffer 1 and the membrane was equilibrated for 5min in lumigen buffer 3. 45ul of NBT solution (75mg/ml in dimethyl formamide) and 35ul of X-Phosphate solution (50mg/ml in dimethyl formamide) were added to 10ml buffer 3 and the resulting "colour solution" was added to the membranes. The membranes were incubated in the dark for 15min to allow the colour precipitate to form. Estimates of labelling efficiency were then made on the basis of comparison to the supplied control labelling reaction. Typically, labelling was at least as efficient as in the provided control reactions.

2.2.2 DNA SEQUENCING

2.2.2.1 Manual dideoxynucleotide DNA sequencing.

2.2.2.1.1 Preparation of single-stranded DNA template.

A single pBluescript-transformed XL-1 Blue colony was inoculated into 50ml 2XYT medium, supplemented with 50υg/ml ampicillin and 50υg/ml tetracycline. VCS helper 'phage (kanamycin resistant) was added to 10^8 pfu/ml. The culture was incubated at 37°C for 2hrs, with shaking, and VCS-producing cells were selected by addition of kanamycin to 70υg/ml. The culture was then grown overnight at 37°C, with shaking. Bacterial cells were separated by centrifugation at 8,000xg for 10min at 4°C. The supernatant was placed in a fresh tube, and 5ml 20% (w/v) Polyethylene Glycol (8000), 2.5M Sodium Chloride added. The 'phage were precipitated on ice for 1hr, harvested by centrifugation at 12,000xg for 5min at 4°C, and resuspended in 400υl TE. The 'phage solution was phenol extracted twice, and the aqueous phase removed to a fresh tube. Single-stranded 'phagemid DNA was Ethanol precipitated,

washed in 80% ethanol, dried *in vacuo* and resuspended in TE. Typically, 1υg of single-stranded DNA was obtained from each 1ml of starting culture. This DNA was used directly in primer annealing reactions.

2.2.2.1.2 Denaturation of double-stranded DNA.

Double-stranded DNA denaturation was carried out by addition of 0.1X volumes of 2M Sodium Hydroxide, 2mM EDTA to 3-5υg of plasmid DNA. The solution was incubated for 30min at 37°C and neutralised by addition of 0.1X volumes of 3M Sodium Acetate (pH 4.8). The denatured DNA was precipitated with 2-4 volumes of ethanol and incubated for 30min at -70°C. The DNA was pelleted by centrifugation in a microcentrifuge at 17Krpm for 15min at 4°C, washed in 80% (v/v) ethanol and dried *in vacuo*. The pellet could then be resuspended in 7υl dH_2O and used in an annealing reaction exactly as for single-stranded template.

2.2.2.1.3 Annealing of template and primer.

Annealing and subsequent sequencing reactions were carried out using Sequenase® version 2.0 DNA sequencing kit. 7υl of template DNA (1υg single-stranded, 3-5υg denatured double-stranded) was mixed with 1υl of sequencing primer (~2pmoles) and 2υl of sequenase reaction buffer (USB). The annealing mix was heated to 65°C for 2min, left to cool slowly to below 35°C and placed on ice.

2.2.2.1.4 Termination reactions.

Four tubes were labelled T, C, G and A. 2.5υl of the appropriate termination mix was added to the labelled tubes (e.g. ddATP-containing mix added to 'A' tube) which were placed at 37°C. Sequenase DNA polymerase was diluted 1:8 in enzyme dilution buffer (USB), and sequenase 5X labelling mix was diluted to 1X. The following components were added to the annealed primer/template mix on ice; 1υl DTT (0.1M), 2υl 1X sequenase labelling mix, 0.5υl ^{35}S-dATP (5υCi) and 2υl diluted

sequenase DNA polymerase. The reaction components were mixed by pipetting and the labelling reaction incubated at room temperature for 2-5min. 3.5υl of labelling reaction was added to each of the four termination tubes, mixed by pipetting and the termination reactions incubated at 37°C for 5min. 4υl of stop solution/loading buffer was added to each termination reaction, mixed by pipetting and the reactions stored on ice until required for loading.

2.2.2.1.5 Polyacrylamide gel analysis of DNA sequencing reactions.

Sequencing gel mix was 6% (w/v) acrylamide (19:1 acrylamide:bis-acrylamide) in 7.7M urea (in 1X TBE). For a 50ml gel, 100υl 25% APS and 100υl TEMED was added to 50ml gel mix and the gel poured between taped, siliconised, glass plates. The 'shark's-tooth' type of comb was used. Gels were run in 1X TBE buffer. Prior to loading, gels were pre-warmed by running at 40 watts (for 40cm x 30cm x 0.4mm gels) for 30min. Termination reactions were denatured at 75°C for 2min and snap-cooled on ice. 3υl of each termination reaction was loaded, in the order T-C-G-A. Gels were run at 40 watts for 2-9hrs (40cm x 30cm x 0.4mm gels), or at 70 watts for up to 16hrs (80cm x 30cm x 0.4mm gels). Following electrophoresis, gels were fixed for 20min in 10% (v/v) Acetic Acid, 10% (v/v) methanol, transferred to blotting paper and dried under vacuum. Autoradiography was carried out to X-ray film, at room temperature, for 5-24hrs.

2.2.2.2 Automated dideoxynucleotide DNA sequencing.

Automated sequencing reactions were carried out using PRISM™ Ready Reaction DyeDeoxy™ dye-labelled terminator cycle sequencing kits (Perkin Elmer) and analysed using an ABI automatic sequencer. Template DNA (250-500ng double-stranded plasmid, 200ng PCR product) in a volume of 11υl, was mixed with 1υl sequencing primer (3.2pmoles) and 8υl Ready Reaction mix (Perkin Elmer, containing labelling mix, dye-labelled ddNTPs & thermo-stable DNA polymerase). The reaction was overlaid with mineral oil and placed in a PCR thermal cycler,

pre-heated to 96°C. Cycling was initiated as follows; 30sec @ 96°C, 15sec @ 50°C, 4min @ 60°C. 25 cycles were performed. The reaction was placed on ice and 2υl 3M Sodium Acetate (pH 4.6), 50υl 50% (v/v) ethanol added. Nucleic acid was precipitated on ice for 10min and pelleted in a microcentrifuge at 17Krpm for 30min at 4°C. The pellet was washed in 70% (v/v) ethanol, dried *in vacuo* and stored at 4°C. The pellet was later resuspended in loading buffer, denatured and analysed on an automatic sequencer.

2.2.3 MANIPULATIONS WITH BACTERIOPHAGE LAMBDA

2.2.3.1 Plating bacteriophage lambda.

Plating cells were prepared as follows. A single colony of host bacterial cells was inoculated into 50ml L-broth, supplemented with 0.4% (w/v) maltose, and grown overnight at 37°C with shaking. Cells were harvested at 2000xg for 10min, resuspended in 20ml 0.01M Magnesium Sulphate and stored at 4°C (up to 3 weeks). 1υl of 'phage stock (typically 1 plaque [~1 X 10^{10} pfu] stored in 1ml SM/50υl chloroform) was added to 100υl plating cells, 100υl adsorption buffer. This adsorption mix was incubated for 10min at room temperature and 20min at 37°C, to enable adsorption and injection. The adsorption mix was then added to 3ml 0.7% top agarose, at 47°C, swirled and poured evenly over L-agar. When set, the plate was covered, inverted and incubated overnight at 37°C.

2.2.3.2 Screening bacteriophage lambda libraries.

Libraries were plated on large square petri dishes, such that one plate contained around 1 X 10^5 plaques. Hybond N^+ nylon membrane was cut to the size of the plate, lowered over the surface of the plate and its position marked with a syringe needle. After 1min, the membrane was peeled off the top agarose, and lowered, plaque side up, into a tray of denaturing solution. After 7min, the membrane was transferred to a tray of neutralising and soaked for 3min. The membrane was rinsed carefully in a

tray of 2X SSC, air dried and fixed by UV cross-linking. Replica membranes were made for each plate. The membranes were hybridised with a gene-specific probe, as for Southern blots, and exposed to X-ray film. Plaques which hybridised with the gene-specific probe were removed from the original plate, using a cut P-200 yellow-tip and stored overnight, at 4°C, in 1ml SM, to allow 'phage to diffuse out of the agar plug. Phage from the plaque were then re-plated at low density to allow selection of well separated plaques. Again, Hybond N^+ membrane lifts were taken and hybridised with a gene-specific probe. Hybridising plaques were selected and stored in 1ml SM, 50ul chloroform, at 4°C.

2.2.3.3 Preparation of bacteriophage lambda DNA.

Liquid lysates were used to prepare 'phage lambda DNA. The 'phage from which DNA was to be prepared were plated out at low density and a single well-separated plaque removed into 300ul adsorption buffer. The plaque was left to diffuse overnight at 4°C. A single colony of host cells was inoculated into L-broth, supplemented with 0.4% maltose, and the culture grown to an OD_{600} of 0.6-1.0. 200ul of these Log-phase cells were added to the 300ul of adsorption buffer containing the diffused plaque. The cells/phage mixture was incubated for 10min at room temperature and then for 30min at 37°C. The adsorbed 'phage were then added to 50ml L-broth (supplemented with 0.1% glucose, 10mM Magnesium Choride) and incubated at 37°C overnight, with shaking. If lysis was incomplete, chloroform was added to 1ul/ml. Cell debris was removed by centrifugation at 8000xg for 10min at 4°C. The supernatant was removed to a fresh tube and 100ul of Qiagen L1 solution added. The lysate was incubated at 37°C for 30min. 10ml of ice-cold Qiagen L2 was added, and the 'phage precipitated for at least 60min on ice. The precipitated 'phage were harvested by centrifugation at >10,000xg for 10min and resuspended in 3ml of Qiagen L3. 3ml of Qiagen L4 was added and the phage coats denatured at 70°C for 20min. The 'phage solution was cooled on ice, 3ml of Qiagen L5 added and precipitated protein removed by centrifugation at >15,000xg for 30min, at 4°C. The supernatant was added onto a Qiagen Tip-100 column (previously equilibrated with

3ml QBT buffer) and allowed to enter completely. The column was washed with 20ml QC buffer and the 'phage DNA eluted with 5ml QF buffer. DNA was precipitated by addition of 3.5ml isopropanol and pelleted in a centrifuge at 15,000xg for 30min, at 4°C. The pellet was washed in 80% (v/v) ethanol, dried *in vacuo* and resuspended in TE. Typically, this method yielded 60-100υg of 'phage DNA. Phage were either stored as plaques in SM/chloroform, or 1ml samples of cell debris-free liquid lysate was supplemented with 50υl chloroform and stored at 4°C.

2.2.4 *IN SITU* HYBRIDISATION TO WHOLE-MOUNT TISSUES

2.2.4.1 Fixation of tissues.

Tissues were dissected in paraformaldehyde/PIPES (PP) solution (50ml; 40ml DEPC dH_2O, 2g paraformaldehyde, 30υl 10M Sodium Hydroxide, 5ml 0.5M PIPES, 100υl 0.5M EGTA [ethylene-glycol-bis{2-aminoethylether}-N,N-tetra acetic acid], 100υl 1M Magnesium Sulphate, pH 6.8). The tissues were transferred to 1ml of Fix solution (1:1 heptane:PP) and allowed to fix for 1-1.5hr, with shaking. The Fix was removed and the tissues treated as follows. The tissues were washed three times for 1min in 9:1 methanol:0.5M EGTA; once for 5min in 7:3 methanol:EGTA; once for 5min in 5:5 methanol:EGTA; once for 5min in 3:7 methanol:EGTA; once for 5min in PP and once for 20min in PP. At this stage, tissues could be dehydrated by serial dehydration and stored in ethanol at 20°C. They must, however, be re-hydrated before proceeding with further steps.

2.2.4.2 Dehydration and re-hydration of tissues.

Dehydration was carried out as follows. Tissues were washed twice for 5min in 1X PBS; once for 5min in 30% (v/v) ethanol; once for 5min in 50% (v/v) ethanol and once for 5min in 70% (v/v) ethanol. The tissues were then stored at -20°C in ethanol. Re-hydration was carried out as follows. Tissues were washed once for 5min in 70%

(v/v) ethanol; once for 5min in 50% (v/v) ethanol; once for 5min in 30% (v/v) ethanol; once for 5min in 1X PBT and once for 30min in 1X PBT.

2.2.4.3 Tissue pre-treatment and hybridisation.

The following steps were all carried out in 1.5ml Eppendorf tubes, at room temperature, using a revolving wheel, unless otherwise stated. The tissues were rinsed three times, for 5min each time, in PBT (1X PBS + 0.1% [v/v] Tween-20) and then treated for 3.5min with 50υg/ml proteinase K (in PBT). Proteinase digestion was stopped by washing twice, for 1min each time, in 2mg/ml glycine. The tissues were washed twice, for 5min each time, in PBT and once, for 20min, in PP. This was followed by six, 5min, PBT rinses. Finally, the tissues were rinsed in 200υl 1:1 PBT:Hybrix (50% [v/v] formamide, 5X SSC, 50υg/ml heparin, 0.1% [v/v] Tween-20, 100υg/ml sonicated, denatured, salmon sperm DNA) for 10min and pre-hybridised in 100υl Hybrix for 60min at 45°C, without shaking. DIG-Labelled riboprobe was ethanol precipitated, pelleted, resuspended in Hybrix and added to the pre-hybridised tissues. Hybridisation was carried out overnight, at 45-48°C, without shaking.

2.2.4.4 Post-hybridisation washes and anti-DIG-AP antibody adsorption.

Following hybridisation, the tissues were washed, for 20min, in 500υl Hybrix at 45°C, then twice, for 20min, in 500υl 1:1 PBT:Hybrix at 45°C. This was followed by a 20min PBT rinse at 45°C and five, 5min, PBT rinses at room temperature. Anti-DIG-AP antibody (@ 75mU/ml) was pre-adsorbed to a second sample of the relevant tissues for 1.5hr (to reduce non-specific background signal) and then added to the probe-hybridised tissues and incubated for 1.5hr at room temperature. The tissues were washed once, for 5min, and then three times, for 20min, in PBT, to remove excess antibody.

2.2.4.5 Detection of anti-DIG-AP antibody.

Tissues were washed three times, for 5min, in NMTT (100mM Sodium Chloride, 50mM Magnesium Chloride, 100mM Tris-HCl [pH 9.5], 1mM levamisole [a potent lysosomal phosphatase inhibitor]). The tissues were then incubated in the dark, for 1-10hr, in 1ml NMTT, containing 3.5υl 50mg/ml X-phosphate, 4.5υl 75mg/ml NBT. Tissues were checked periodically, for colour development and the colour reaction stopped by 3 PBT washes.

2.2.5 POLYMERASE CHAIN REACTION

2.2.5.1 Reaction conditions and optimisation.

Polymerase chain reactions (PCRs) were carried out using Promega Taq (*Thermus aquaticus*) thermo-stable DNA polymerase and 10X PCR reaction buffer (500mM Potassium Chloride, 100mM Tris-HCl [pH 8.3], 0.01% [w/v] gelatin). Reactions were performed in a final volume of 50υl and were overlaid with 50υl of mineral oil. As a starting point, reactions were set up as shown below;

3υl 25mM Magnesium Chloride
1υl 10mM dNTP's (dATP, dTTP, dCTP, dGTP)
5υl 10X PCR Reaction buffer
0.5υl Each oligonucleotide primer (50pmol/υl)
2υl Template
2υl Taq Polymerase (1.25 U/υl)
36υl dH2O

50υl Total

Cycling conditions are essentially divided up into 3 phases; denaturation (@ 94-96°C), annealing (@ 4 degrees below the primer annealing temperature [Tm])

and extension (@ 72°C). The approximate Tm of any given primer was calculated using the equation; Tm=2 x (Number of A & T nucleotides) + 4 x (number of G & C nucleotides). For Promega Taq polymerase, the extension phase was estimated to proceed at the rate of 1-2kb per min. Thus, for amplification of a 1kb product, using primers of Tm=60°C, the following cycling conditions might be used;

Stage 1 (1 cycle): Hot Start. Reactions (excluding Taq) placed in thermal cycler and heated to 95°C for 2min. Temperature held at 80°C, while Taq is added.

Stage2 (25 cycles): denaturation @ 94°C for 15sec; annealing @ 56°C for 15sec; extension @ 72°C for 1min.

Stage 3 (1 cycle): Final extension @ 72°C for 10min.

The hot-start method was used to prevent non-specific priming during the initial climb to 94°C, in stage 2.

These conditions represent a starting point and each set of primers and template was optimised to minimise non-specific products and maximise yield. In general, all phases were kept as short as possible to maximise enzyme life, and denaturation was done at 94°C, rather than 95°C-96°C, for the same reason. The production of non-specific products was minimised in the following ways; reduction of annealing/extension times, reduction of cycle number, increasing annealing temperature, reduction of Magnesium Chloride concentration, reduction of dNTP concentration and reduction of Taq concentration. PCR products were analysed on agarose gels.

2.2.5.2 PCR of reverse transcription products.

1ul of Oligo dT (1mg/ml) was added to 10ul of total cellular RNA (1mg/ml) and incubated at 70°C for 10min, then snap-cooled on dry ice. The following components

were added; 4υl 5X 1st strand cDNA synthesis buffer, 2υl 0.1M DTT, 1υl 10mM dNTPs, 1υl DEPC dH_2O, 0.3υl RNAguard™ (Pharmacia), 1υl Superscript II™ reverse transcriptase (200U/υl). The reverse transcription reaction was incubated at 37°C for 60min, made up to 100υl with DEPC dH_2O and phenol/chloroform extracted once. 5υl of reverse transcriptase reaction was used in each PCR reaction, which were carried out as described above.

2.2.5.3 Rapid amplification of 3' cDNA ends (3' RACE).

Single-stranded cDNA was synthesised as described above. PCRs were carried out using an oligodT primer and a gene-specific primer (polymerising towards 3' end of transcript). In this way it is possible to amplify the 3' end of a cDNA, rapidly, from total cellular RNA.

2.2.5.4 Rapid amplification of 5' cDNA ends (5' RACE).

Single-stranded cDNA was synthesised, as described above. However, instead of phenol/chloroform extraction, the reaction was stopped via inactivation of the reverse transcriptase by heating to 55°C for 5min. 1υl of RNAase H (2U/υl) was added and the reaction was incubated at 55°C for 10min, then placed on ice. cDNA was purified away from primers and dNTPs using Qiaex DNA affinity matrix, as described above. 13υl of the total 40υl of cDNA, was removed to a fresh tube, denatured at 70°C for 10min and snap-cooled on ice. A PolyA tail was added to the 5' ends of the single-stranded cDNA molecules, as follows. The following components were added to the cDNA on ice; 4υl 5X Terminal transferase (TdT) reaction buffer, 2υl 2mM ATP, 1υl TdT (10U/υl). The terminal transferase reaction was incubated at 37°C for 10min and stopped at 70°C for 10min. 5υl of the stopped terminal transferase reaction was used in PCRs with oligodT and a gene specific primer (polymerising towards 5' end of transcript) to amplify the 5' end of transcripts represented in the single-strand cDNA pool.

2.2.6 PROTEIN ANALYSIS METHODS

2.2.6.1 Analysis of proteins by polyacrylamide gel electrophoresis.

Polyacrylamide gels for protein analysis were 10% acrylamide and were made up as follows; 13.3ml 30% (w/v) acrylamide, 10ml 1.5M Tris-HCl (pH 8.8), 0.2ml 20% (w/v) SDS, 16.1ml dH_2O. 400υl 10% (w/v) APS and 16υl TEMED were added and the gel was poured between glass plates. 1ml of H_2O-saturated butanol was used to overlay the gel, until polymerisation was complete. A 5% stacking gel was made up as follows; 1.7ml 30% (w/v) acrylamide, 1.25ml 1M Tris-HCl (pH 6.8), 50υl 20% (w/v) SDS, 6.85ml dH_2O. 100υl 10% (w/v) APS and 10υl TEMED were added and the gel was poured so as to overlay the 10% resolving gel. A comb was inserted and the gel was left to polymerise. Protein samples were prepared for loading by addition of an equal volume of 2X protein loading buffer and boiling for 3min. Gels were run in Tris/glycine buffer (25mM Tris, 250mM glycine, 0.1% [w/v] SDS, pH 8.3), at voltages recommended by the gel apparatus manufacturer. Following electrophoresis, gels were stained using the ISS Pro-Blue system (Integrated Separation Systems). Gels were fixed in 12% TCA for at least 60min. The TCA was then removed and the gel soaked in pretreatment solution (20ml Reagent A, 80ml dH_2O) for 60min. The gel was then soaked in staining solution (16ml Reagent A, 1.6ml Reagent B, 20ml methanol, 64ml dH_2O) until protein bands were apparent (>1hr). Excess stain was removed in 25% (v/v) methanol. The gel was photographed and stored in 20% (v/v) ethanol at room temperature.

2.2.6.2 Purification of GST-fusion proteins.

The open reading frame of interest was cloned into an expression vector of the pGEX series of glutathione-S-transferase (GST) fusion protein expression vectors. This construct encoded a protein containing a region of GST which binds to its substrate, glutathione. Thus, the protein can be purified using glutathione immobilised upon an agarose matrix. A single pGEX-transformed colony was

inoculated into 100ml L-broth, supplemented with 100ug/ml carbenicillin and grown at 37°C, with shaking, until an OD_{600} of 0.6-1.0 was reached. Fusion protein expression was induced by addition of IPTG to 0.1mM and the culture grown for a further 4 hours. Cells were harvested by centrifugation at 5000xg for 10min, at 4°C, and resuspended in 20ml ice-cold 1X PBS. The cells were lysed by brief sonication on ice and proteins solubilised by addition of Triton X-100 to 1% (v/v). Insoluble protein and cell debris was removed by centrifugation at 10,000xg for 5min, at 4°C. 1ml of 50% (v/v) glutathione-agarose slurry (Sigma) was added and gently mixed for 30min. The slurry was collected by a 10sec centrifugation at 500xg, washed three times in 50ml ice-cold 1X PBS and resuspended in 1ml ice-cold 1X PBS. GST-fusion protein was eluted in 1ml 50mM Tris-HCl (pH 8.0), 5mM glutathione, by mixing gently for 2min. Glutathione-agarose slurry was pelleted by a 10sec centrifugation at 500xg and the eluate removed to a fresh tube. Three further elutions were performed and the eluates stored at -70°C.

2.2.6.3 Western blot analysis of protein SDS polyacrylamide gels.

Following electrophoresis of protein samples (2.2.6.1) the gel was electroblotted to Hybond C nitrocellulose membrane. A sheet of Hybond C was cut to the size of the gel and placed over it. The gel and membrane was sandwiched between 4 sheets of blotting paper and the whole assembly placed between two sheets of sponge. This was then inserted into a Biorad electroblotting apparatus, with the Hybond membrane facing the positive electrode. The tank was filled with Tris/glycine/methanol buffer (1 litre: 9g Tris, 432g glycine, 3g SDS, 600ml methanol) and blotted overnight at 15-20mA (~6v). The membrane was removed from the gel and blocked in TBS (10mM Tris-HCl [pH 8.0], 100mM Sodium Chloride, 0.5% [v/v] Tween-20) + 5% (w/v) fat-free milk powder, 0.02% (w/v) Sodium Azide, for 1-2hr at room temperature. Primary antibody (e.g. Sigma anti-GST rabbit IgG) was added to the membrane, at 1-10ug/ml in TBS + 5% (w/v) fat-free milk powder, 0.02% (w/v) Sodium Azide and incubated for 2hr at 4°C. The membrane was washed five times, for 5min each time, in TBS and secondary

antibody (e.g. Promega anti-rabbit IgG Horseradish peroxidase [HRP] conjugate) was added (@ 1υg/ml in TBS + 5% [w/v] milk powder) and incubated for 1-2hrs at room temperature. Excess antibody was removed by five, 5 min, washes in TBS. Anti-rabbit IgG HRP conjugate was detected using the isoluble peroxidase substrates Diaminobenzidene tetrahydrochloride (DAB) and Urea Peroxide. One pre-prepared tablet of each chemical (Sigma) was suspended in 1-20ml of TBS and poured over the membrane. The membrane was incubated at room temperature, with shaking, until colour precipitate was formed (typically within 15min).

2.2.6.4 Kinase assays of crude bacterial extracts.

A pGEX expression construct encoding the protein to assayed for kinase function was transformed into XL-1 blue cells. A single colony of these cells was inoculated into 50ml 2XYT medium, supplemented with 100υg/ml carbenicillin. The culture was grown at 37°C, with shaking, until an OD_{600} of 0.6-1.0 was reached. Protein expression was induced by addition of IPTG to 1mM, and the culture was grown for a further 3-4hr at 30°C, with shaking. A 1ml sample was taken, and the cells harvested by brief centrifugation in a microcentrifuge. The cells were washed in 1ml 1X PBS and resuspended in 100υl 1X PBS. Lysis was performed by freeze-thawing four times (liquid Nitrogen <–> 42°C water bath) and brief sonication on ice. A 25ul aliquot was removed to a fresh tube, containing a 1.5υl cocktail of proteinase inhibitors (1:1:1 ratio of 100υg/ml leupeptins, 100υg/ml pepstatin A, 100mM PMSF). 0.3υl of ^{32}P-γ-ATP (3υCi) was added to the crude protein extract and the kinase assay reaction incubated for 60min at 25°C. Reactions were analysed using SDS PAGE and autoradiography. Control reactions were carried out in parallel with extracts from cells expressing pGEX vector alone and with un-induced cells. In addition, samples were taken, to which ^{32}P-γ-ATP was not added, but which were otherwise treated exactly as the assay samples. These samples were analysed by SDS PAGE and the gels were stained, to examine expression efficiency and integrity of expressed proteins used in the assay.

CHAPTER 3

CLONING OF A GENE ENCODING SEX-SPECIFIC TRANSCRIPTS IN *DROSOPHILA MELANOGASTER*

3.1 INTRODUCTION

The phenotypic effects of the sex determination hierarchy genes; *Sxl*, *tra*, *tra-2* and *dsx* indicates that the majority of genes controlling the sexual identity of the fly are under control of this hierarchy. It is clear that the *yp*'s in the fat body remain under continuous control of *dsx* throughout development, while, in the ovary, the control of these genes appears to be handed over to ovary-specific factors early in development. The mode of action of *Sxl*, *tra*, *tra-2* and *dsx* is to cause their target genes to produce sex-specific transcripts. Since it is clear that the majority of genes responsible for sex-specific development are under control of the sex determination hierarchy, it is reasonable to suppose that a significant proportion of these, as yet uncharacterised, sex differentiation genes are likely to give rise to male-specific or female-specific transcripts.

As discussed earlier, previous differential screens have only isolated genes which express gonad-specific transcripts. The high abundance of *yolk protein* gene transcripts means that these genes are often re-isolated in differential screens. In an effort to deal with these problems, a differential screen was carried out in this lab using only flies which had first had their gonads dissected out. Thus, much of the *yolk protein* and *chorion* gene transcripts were eliminated from the screen. To remove those *yolk protein* transcripts which are also produced in the female fat body, polyA$^+$ RNA prepared from this tissue was firstly hybridised to cloned *yolk protein* DNA and passed through hydroxyapatite, which selectively binds double stranded nucleic acid molecules. These precautions successfully eliminated *yolk protein* transcripts from the differential screen, since no *yp*-derived recombinants were isolated. Radiolabelled cDNA was made from mRNA prepared from male and female carcasses. This was used to differentially screen a Charon 4 bacteriophage lambda ('phage λ) genomic library, as shown in figure 3.1. From this screen, eight putative female-specific and seven putative male-specific recombinant 'phage were isolated. In this chapter, I describe the initial analysis of one of the putative female-specific 'phage, λfs(1). This recombinant was isolated on the basis of

preferential hybridisation to female cDNA, although some hybridisation to male cDNA was also seen.

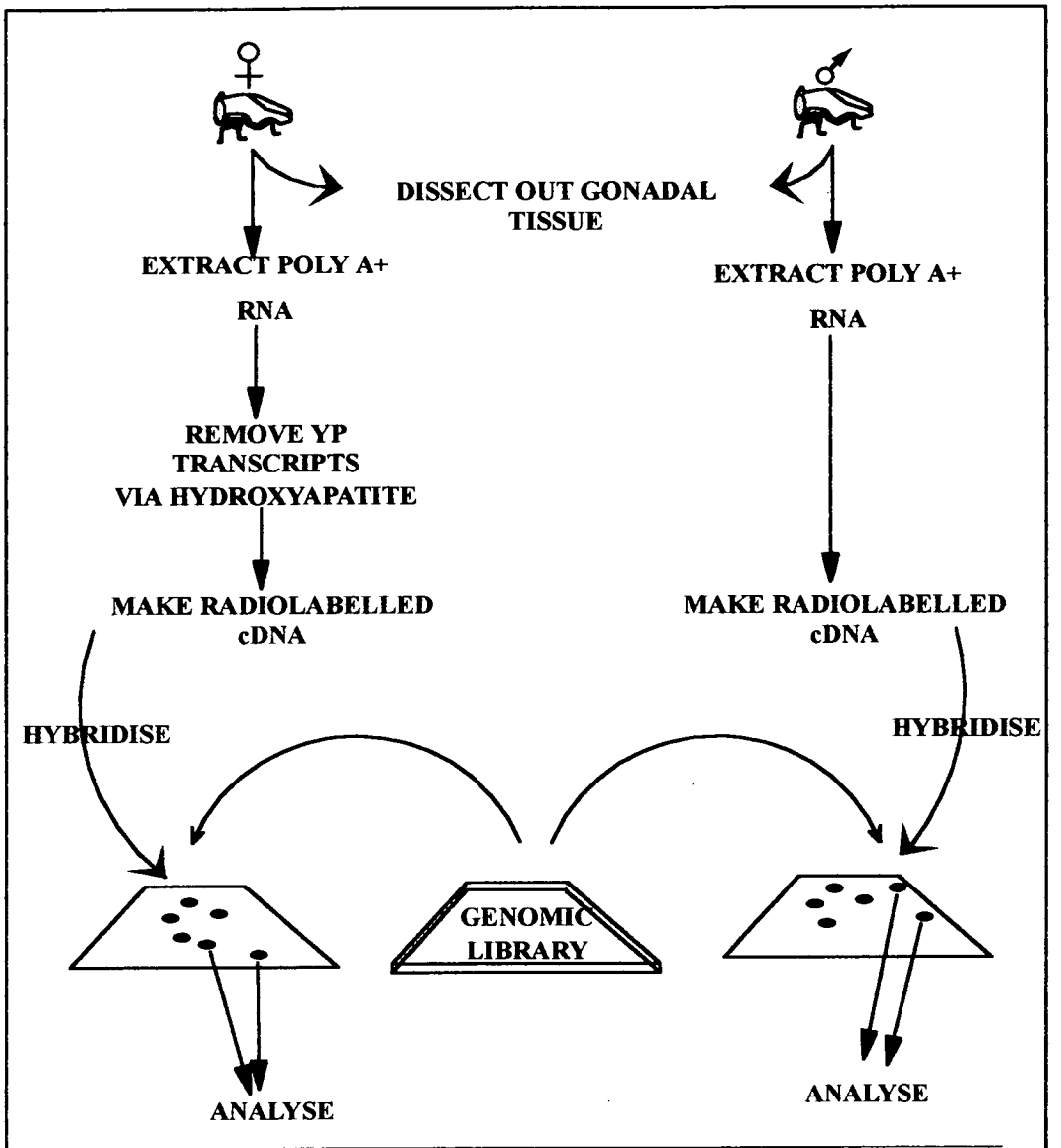


Figure 3.1
Diagram showing strategy of differential genomic library screen. Only non-gonadal tissue was used for the screen. PloyA$^+$ RNA was extracted from male and female OrR carcass tissue. Female RNA was hybridised with *yp* cDNA and then passed over a hydroxyapatite affinity column to remove double stranded molecules. Radiolabelled cDNA was synthesised from the *yp*-free female RNA and male RNA. This cDNA was used as a probe to screen replica filters lifted from a plated bacteriophage λ genomic DNA library. Recombinants which hybridised with cDNA from one sex only were isolated for further analysis.

3.1.1 PRELIMINARY WORK - O. CLAIRE GILMAN

Initial characterisation of λfs(1) (together with four other putative female-specific 'phage) was the subject of a Ph.D. project carried out by O. Claire Gilman (thesis submitted 1991) and is summarised briefly below.

After checking that λfs(1) did not hybridise to cloned *yp* DNA and was therefore not a reisolate of these genes, the 'phage was restriction mapped to reveal the pattern of *Eco*RI sites shown in figure 3.2.

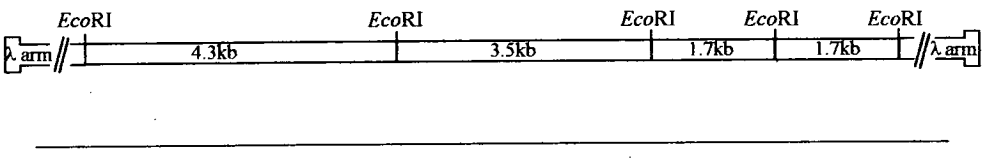


Figure 3.2
*Eco*RI restriction map of bacteriophage λ recombinant fs(1) [taken from Gilman, 1991].

To determine the cytogenetic location of any genes contained in λfs(1), *in situ* hybridisation to third instar larval salivary gland polytene chromosomes was carried out (Robert Saunders, University of Dundee). The 4.3kb *Eco*RI restriction fragment of λfs(1) was subcloned into the *Eco*RI site of pGemini-1 (pGEM-1), and this plasmid was used to synthesise a probe for the *in situ* hybridisation. The cytological position detected by this probe was determined to be 061C1-3, lying at the distal end of the left arm of chromosome 3. The Flybase database was searched for existing mutations in this region which result in sex-specific phenotypes, but none were isolated.

Reverse Northerns were then carried out with radiolabelled cDNA from male and female flies as probes. Of the four *Eco*RI restriction fragments, the 3.5kb fragment hybridised to both male and female cDNA and the 4.3kb fragment hybridised only to female cDNA. This indicates that there is at least one gene present in λfs(1), giving rise to both female-specific and non sex-specific or male-specific transcripts.

However, the cDNA used as a probe for these reverse Northerns was derived from whole flies and so does not rule out the possibility that the sex-specific transcripts are of gonadal origin. Northern analysis using the same 4.3kb *Eco*RI fragment in pGEM-1 suggested the presence of a female-specific transcript, as would be expected from the reverse Northern data, but was not particularly clear (data not shown).

3.1.2 PRELIMINARY WORK - M. TODMAN

Further analysis of the 4.3kb *Eco*RI λfs(1) fragment was carried out by Martin Todman (Research Assistant, 1991-1992). The fragment was recloned into the *Eco*RI site of the sequencing vector pBluescript-SK$^+$ (This subclone henceforth referred to as λfs(1)$^{4.3}$) and was used as a probe to screen a λGT11 'phage λ adult body cDNA library. Three recombinant 'phage which hybridised with λfs(1)$^{4.3}$ were isolated (cDNAa, cDNAb and cDNAc). These cDNAs contained no internal *Eco*RI sites and so could be separated from their lambda arms by *Eco*RI digestion. Thus, the entire cDNAs were cloned into the *Eco*RI site of pBluescript-SK$^+$. Sequence analysis of the three subcloned cDNAs revealed that two of them were either truncated (cDNAb) or contained artefacts (cDNAc). One 3.0kb cDNA (cDNAa) was apparently full length, having both a polyA tail and a long open reading frame. The pBluescript subclone containing cDNAa (cDNAa^{SK+}) was used to probe a Northern blot of male and female total RNA. Hybridisation was seen to a large female carcass-specific transcript of around 4.5kb (figure 3.3). An ovary-specific transcript of around 3.5kb and a very faint male-specific 3.0kb band were also seen but no non sex-specific transcripts were revealed by this analysis. Thus, the gene which produces the transcript represented by cDNAa fulfils one of the criteria for a candidate non-gonadal sex differentiation gene. Namely, the production of non-gonad sex-specific transcripts. The second criterion; that these transcripts be under the control of the sex determination hierarchy genes, remained to be satisfied.

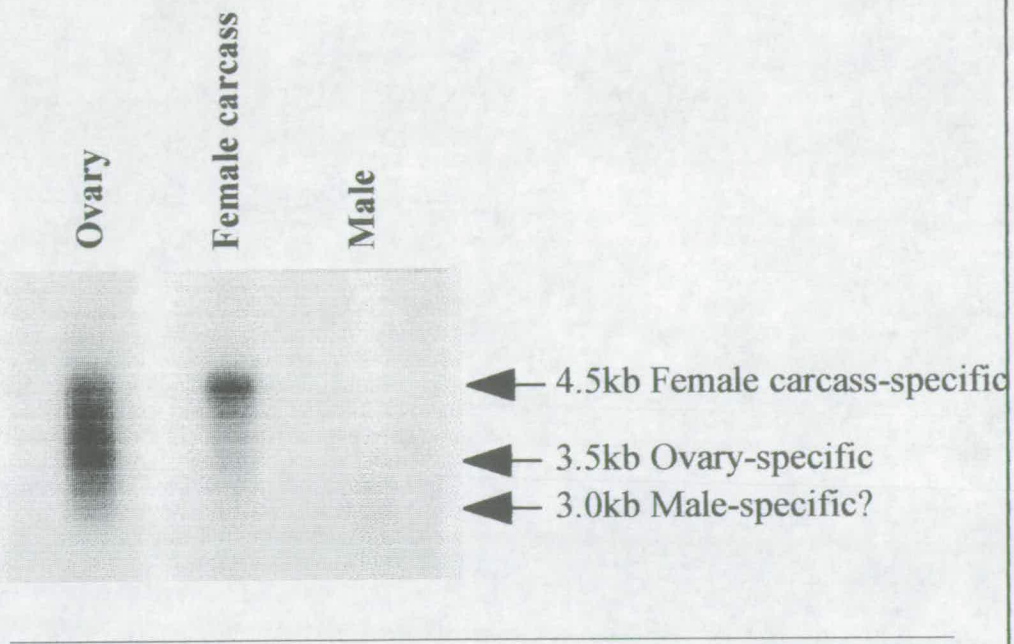


Figure 3.3
Autoradiograph of a Northern blot prepared from hand-dissected OrR tissues. Approximately 20υg of total RNA was loaded per lane. The filter was hybridised with a probe synthesised from cDNAa^{SK+}. A 4.5kb female carcass-specific transcript and a 3.5kb ovary-specific transcript are apparent. There also appears to be a very faint 3.0kb male-specific transcript. This filter was not available for a loading check to be carried out.

The relation of cDNAa to λfs(1)$^{4.3}$ as revealed by restriction mapping is shown in figure 3.4.

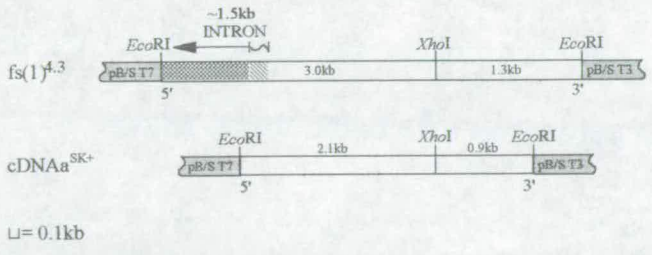


Figure 3.4
Partial restriction maps of cDNAa^{SK+} and fs(1)$^{4.3}$. The orientation of these pBluescript SK$^+$ inserts is shown with reference to the T3 and T7 promoters present at either end of the vector multiple cloning site. Partial sequence data indicates that around 1.5kb at the extreme 5' end of fs(1)$^{4.3}$ consists of intron sequence (hatched area), although the boundaries of this intron were not precisely delimited (indicated by semi-hatched area).

Although not complete at the time, partial sequence data showed that around 1.5kb at the 5' end of $\lambda fs(1)^{4.3}$ was not homologous to cDNAa, indicating the presence of a large intron somewhere in the middle of the cDNA. To try to isolate the remainder of the gene, $cDNAa^{SK+}$ was used as a probe to screen a 'phage λ "FixII" genomic library (Stratagene-see Materials & Methods). Five recombinant 'phage were isolated (designated Fix1, Fix2, Fix4, Fix5 & Fix7) which hybridised with $cDNAa^{SK+}$. These were not analysed further at the time, but will be discussed later in this chapter.

3.2 RESULTS

3.2.1 TRANSCRIPTS PRODUCED FROM *STK61*

To confirm the Northern results obtained by Martin Todman, more Northern blots were carried out. To produce a probe of as high a specificity as possible, it was preferred that the cDNAa insert be purified away from its pBluescript-SK$^+$ vector prior to labelling. However, when cDNAa^{SK+} is digested with *Eco*RI, both the cDNAa insert and the pBluescript-SK$^+$ vector are almost exactly 3.0kb, making separation on an agarose gel impossible. To circumvent this problem, a pBluescript-SK$^+$ subclone of one of the truncated cDNA isolates, cDNAb was used. This truncation was isolated in the cDNA screen performed by Martin Todman, and is an *Eco*RI fragment consisting of the 3' 1557bp of cDNAa. This 1.5kb *Eco*RI fragment was gel purified, radiolabelled, and used to probe a Northern blot of total RNA from whole males, female carcasses (whole flies with gonads removed) and ovaries (figure 3.5).

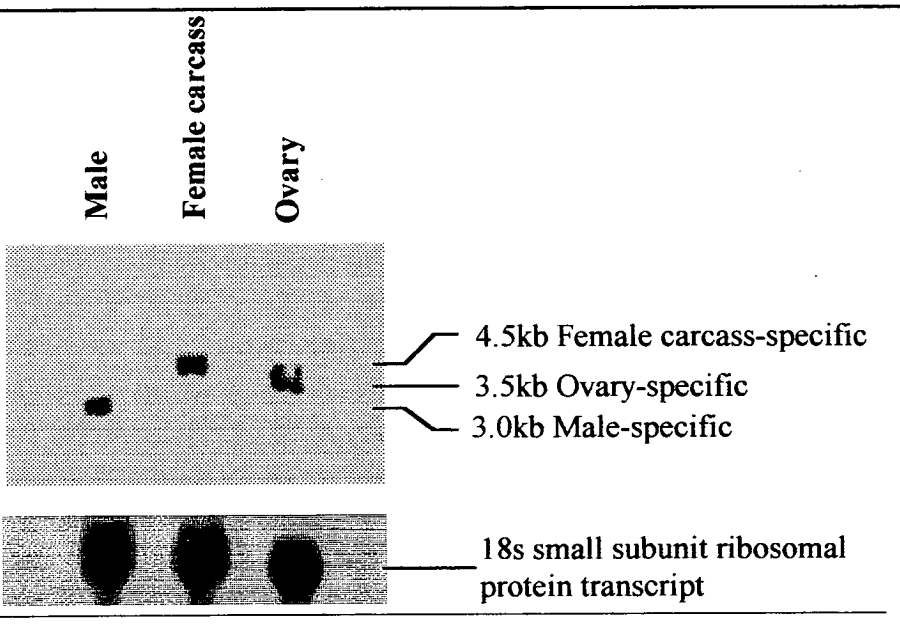


Figure 3.5
Autoradiograph of a Northern blot prepared from hand dissected OrR tissues. Approximately 20ug of total RNA was loaded per lane. The filter was hybridised with a probe synthesised from cDNAb. A 4.5kb female carcass-specific transcript, a 3.5kb ovary-specific transcript and a 3.0kb male-specific transcript are apparent.

The Northern shown in figure 3.5, confirms the presence of a 4.5kb female carcass-specific transcript and a 3.5kb ovary-specific transcript. A male-specific transcript is also clearly visible and appears to be of around 3.0kb in size. The earlier reverse-Northern data suggested the presence of a common transcript as well as a female-specific transcript. Thus the observation of a male-specific transcript on Northern blots is not unexpected. The fact that the male transcript appears to be of around the same size as cDNAa, suggests that cDNAa may represent this transcript. To investigate this, and to further characterise the regulation of the gene, an attempt was made to isolate more cDNAs representing transcripts from this gene.

3.2.2 ISOLATION OF FURTHER cDNAs

A NM1149 'phage λ adult body cDNA library (courtesy Prof. M. Ashburner-see Materials and Methods) was screened using the same cDNAb probe that was used for the Northern in figure 3.5. This probe comprises the 3' 1.5kb of cDNAa and hybridised to 9 'phage λ recombinants. These 'phage were isolated and DNA prepared from them. The NM1149 library used for this screen was constructed by addition of linkers containing restriction sites to the double stranded cDNA. The sequence of these linkers was such that a *Eco*RI site is added to the 5' end of the cDNA and a *Hin*dIII site to the polyadenylated 3' end. The cDNA's were then directionally cloned into the *Eco*RI and *Hin*dIII sites of the NM1149 polylinker. Thus, *Eco*RI/*Hin*dIII double digestion of the cDNA library isolates liberates the cDNA from the vector arms and reveals any internal *Eco*RI and *Hin*dIII sites. DNA from the 9 isolates was digested with *Eco*RI and *Hin*dIII, and the resulting pattern of restriction fragments showed that only 2 of these isolates were both unique and large enough to represent the transcripts observed on Northern blots (data not shown). Hereafter, these cDNA's are referred to as cDNA1 and cDNA11. Figure 3.6(a) shows an *Eco*RI/*Hin*dIII digest of cDNA's 1 & 11. cDNA 11 consists of 1 *Eco*RI/*Hin*dIII fragment of 1.9kb and 2 *Hin*dIII fragments of 1.0kb and 1.4kb. cDNA1 consists of 1 *Eco*RI/*Hin*dIII fragment of 0.9kb and 2 *Hin*dIII fragments of 1.0kb and 1.4kb. The

*Hin*dIII fragments and *Eco*RI/*Hin*dIII fragments were differentiated using *Eco*RI and *Hin*dIII single digests (data not shown).

To confirm that cDNAs 1 and 11 represented transcripts from the same gene as cDNAa, *Eco*RI/*Hin*dIII digests of the cDNAs were run out on an agarose gel, Southern blotted to nylon membrane and probed with a random-primed probe made using cDNAb. The results of this are shown in figure 3.6(b). The 1.0kb and 1.9kb fragments of cDNA11 hybridise to this probe, as do the 0.9kb and 1.0kb fragments of cDNA1. Thus, cDNAs 1 and 11 do represent transcripts from the same gene as cDNAa. The fact that the 1.4kb *Hin*dIII fragments of cDNA1 and cDNA11 did not hybridise with cDNAb suggests that cDNA11 and cDNA1 may be derived from a differentially processed variant of the transcript represented by cDNAa.

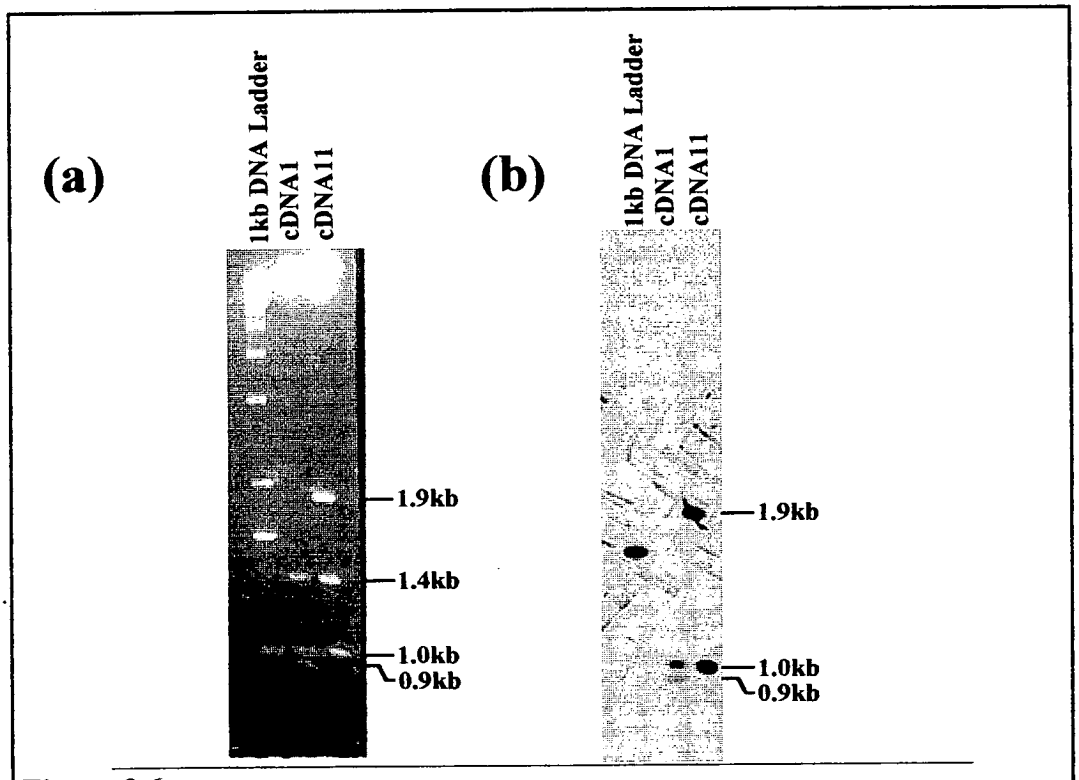


Figure 3.6
(a) 1.0% Agarose gel showing *Eco*RI/*Hin*dIII restriction digests of cDNAs 1 and 11. cDNA11 contains one *Eco*RI/*Hin*dIII fragment of 1.9kb and two *Hin*dIII fragments of 1.4kb and 1.0kb. cDNA1 contains one *Eco*RI/*Hin*dIII fragment of 0.9kb and two *Hin*dIII fragments of 1.4kb and 1.0kb. (b) Autoradiograph of a Southern blot filter made from the gel shown in figure 3.6(a). The Filter was hybridised with a probe synthesised from cDNAb. The 1.9kb and 1.0kb fragments of cDNA11 hybridise with this probe, as do the 0.9kb and 1.0kb fragments of cDNA1.

cDNAs 1 & 11 were subcloned into pBluescript-SK$^+$ using a shotgun cloning method. The 'phage containing the cDNA's were digested either with *Hin*dIII alone, or double digested with *Hin*dIII/*Eco*RI. pBluescript-SK$^+$ was digested with the corresponding enzymes and then treated with Shrimp Alkaline Phosphatase to prevent religation of the vector to itself. Cut/phosphatased vector was then added to the 'phage digests and ligated. An aliquot of these ligations was then used to transform XL-1 Blue cells. Transformed cells were plated on X-Gal/IPTG-containing plates to allow isolation of cells containing recombinant pBluescript via blue/white colour selection. DNA was prepared from white colonies and analysed by restriction digestion with *Hin*dIII and *Hin*dIII/*Eco*RI. In this way, constructs containing all 3 fragments of both cDNA's were generated.

3.2.3 SEQUENCE ANALYSIS OF cDNAs

The insert DNA in these constructs was completely sequenced using chain termination dideoxy sequencing (Materials and Methods). cDNAa^{SK+} was also re-sequenced (partially sequenced by M. Todman.) to clear up ambiguities and false frame shifts in the long open reading frame of the cDNA. The relationship between cDNA a,1 and 11, as revealed by this data, is shown in figure 3.7.

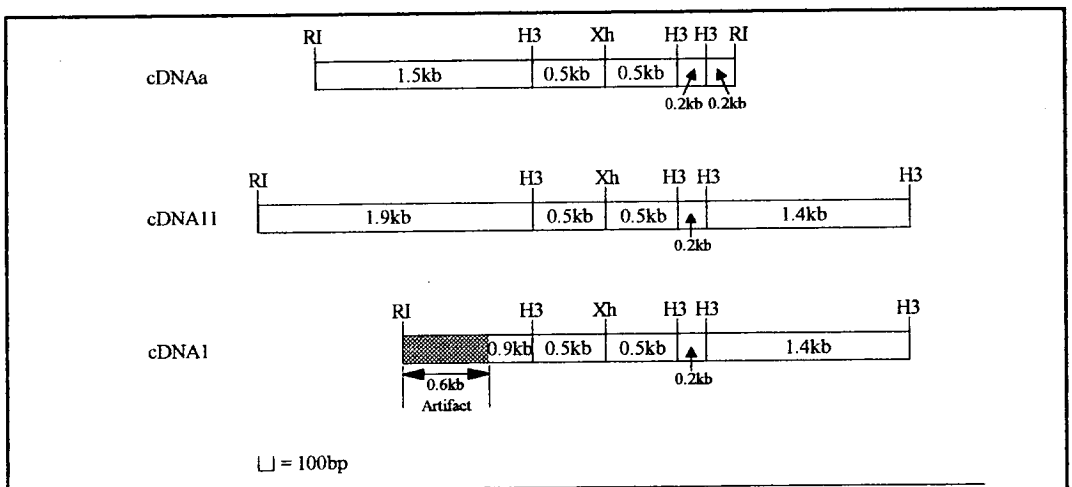


Figure 3.7
Diagram showing partial restriction maps of cDNAs a, 1 & 11. All *Eco*RI, *Hin*dIII and *Eco*RI/*Hin*dIII fragments were subcloned into pBluescript $^{SK+}$, as described in the text. The extreme 5' 0.6kb of cDNA1 consists of the 3' end of a known *Drosophila* ribosomal protein transcript, including its polyA tail. H3=*Hin*dIII, RI=*Eco*RI, Xh=*Xho*I.

The 1.0kb and 1.4kb fragments of cDNAs1 and 11 were shown to be identical in both cDNAs. A 0.2kb *Hin*dIII fragment was discovered as a coligation in several subclones from both cDNAs 1 and 11 which was identical in sequence to the 0.2kb *Hin*dIII fragment of cDNAa. This fragment had not been previously seen clearly on agarose gels due to its small size. It was also seen that cDNA1 is in fact a truncated version of cDNA11, containing 0.6kb of the 3' end of a transcript from a known *Drosophila* ribosomal protein gene at its 5' end. Therefore, cDNA1 was not analysed any further.

The complete sequence of cDNA11 is shown in figure 3.8. The sequence of cDNAa is entirely contained within the sequence of cDNA11 and the 5' and 3' ends of cDNAa are indicated on the cDNA11 sequence. Also indicated is the long open reading frame and the position of all primers used for sequencing and PCR analysis. cDNAs a and 11 differ in length at both their 5' and 3' ends. The addition of a 7-methylguanosine cap to the 5' end of mRNA means that full length cDNAs have a guanine nucleotide at their 5' ends which is not encoded in the transcription unit. By this criterion, neither cDNAa or cDNA11 appear to be entirely full length at their 5' ends. It may be that the full length transcripts represented by cDNAa and cDNA11 share a common 5' end. However, the possibility of alternate promoters cannot be ruled out at this stage. The 3' ends of both cDNAs are complete, having polyA tails and the correct upstream polyadenylation consensus sequence, as shown in figure 3.8. Thus, the transcripts represented by cDNAa and cDNA11 utilise alternate polyadenylation sites.

Figure 3.8

DNA sequence of cDNA11. The sequence of cDNAa and cDNA11 is identical apart from the 5' and 3' ends, which are shorter in cDNAa than in cDNA11. The 5' and 3' ends of cDNAa are indicated on the cDNA11 sequence. The positions of all oligonucleotide primers used for sequencing and PCR analysis are shown. Primers which have the same 5' –> 3' direction as the cDNA11 sequence have been given letter designations (A-G). Those primers which face in the 3' –> 5' direction, relative to cDNA11, are designated by numbers (1-7) and have the complementary sequence to that shown for cDNA11. Also indicated are the positions of intron/exon boundaries and any relevant consensus sequences.

Primer A

```
1     AGTGCTGTAG  TGGTGCGATT  AAATAATACT  CCACAAAAGG  CCAAATAAAT
51    TACATAAAAT  CTAAATCGGA  ATAAAAATAA  GTGTATTCTC  CCTCCGTGTT
101   GTGTTCAAAG  TATTTAACCG  ATTGTTTTCA  TTAGTCAAAT  TAATGTAGTA
151   CAACGATGAG  TGTGAGCCCA  GTTTCCTAGA  AAAATAAGAG  GGCATAAAAA
201   ATAACAGACA  CATACAGCGT  CTCGTTTCTT  ATAAATTTTC  CTACTGCGTC
251   GGAATTATTG  TCTTGATTAT  TGTTGTTTCT  GGCGAACTTT  TGTGATTTGT
301   TTCGAGAAAC  ATACTGCGGC  GGTTTCATAA  TTGTGCAAAT  CAAGGGAAGC
                                                    Primer 1
      cDNAa 5' end
351   ACAATACGAA  TGTTAAGAAT  CAAAGCTAAG  AATTAAATAA  AATTAAAGCC
401   GCACACATAC  ATATACACAG  GCACGCGCAG  AGTCGAAAAA  ATACGCGCGC
            Primer 2
451   TCGCTCTGTA  CAGTGTGTGC  GTGTACGCGG  TGGCTGGCTC  TTCTTCTTCA
                                    TCE 1
501   TTCGTGTGCC  CATTGGAATT  TGTACCCCAA  ATCGAAATTA  CAACATAGAC
551   AAACAAAGAG  CGCCCGTCTG  CCCCAGCACC  TAAACATCAA  CGGTACACAG
                                        Intron 1
601   CAACAGCTCC  AATTGCCAGG  CAGCGGCGCT  AGCGGAATCG  CAGCGGCAGC
651   GTAATCACGG  TAGCATCGGA  TTGCGGCGAA  AACTGCAGCA  GTAACGGCAC
                    TCE 2                           Primer B
701   CGAGCATCAG  CAGCACTTCA  ACATTGCTAC  CACCACAGCA  ACTTCGGCGA
                                ORF Start
751   CAGAGGCAAC  AATACCGGCT  ATGGCCAAGG  AGAAAGCATC  AGCAACAGTG
                                Primer 3
801   TCCTTGGGCG  AGTCGAATTT  CAGAGATATC  AACCTAAAAG  ACTTGGCCGT
851   GGTCGTCGAA  GCGGCATCGC  GGCTGCACCA  CCAGCAAAAC  GTATGCGGCT
901   GCGGAGCGGT  ATCGTCTACT  GAGAACAACA  ACAACAGTCG  CTACGGCAGC
951   AGCAAGTATC  TGACAAACGG  CCACACGTCG  CCTTTGGCGG  CAGCGGTTGC
1001  TAGCAACAGT  TCGTCGGTGG  CCACGACACC  GCATTGCAGA  ATGTTGCACA
```

(continued...)

1051 ACTGCAGTCT GCAGCAGTAC CAGAATGACA TAAGGCAGCA GACGGAGATA

1101 TTGGACATGT TGCGGCACGA GCATCAGCAG GGCTACCAGT CGCAGCAACA

1151 GCAACAACAG CCGCAGCAGC AGCAGGAACA ACAGCAGCAG CAGGAGCAAT

1201 CGCAGCAGCA GCAACAGCTA CAGAATCCTG CGCCCAGGAG GTCTCCGAAT

1251 GATTTCATTT TCGGTCGTTA CATAGGCGAG GGCAGCTATA GCATAGTTTA

1301 TCTGGCCGTG GATATACACT CTCGCCGCGA GTATGCAATT AAAGTATGCG

Intron 2

1351 AGAAGCGGCT GATCCTGCGC GAACGGAAGC AGGACTACAT CAAGCGTGAA

1401 CGCGAGGTGA TGCACCAGAT GACCAACGTT CCCGGCTTCG TAAACCTGTC

1451 GTGCACCTTC CAGGACCAGC GTTCTCTTTA CTTTGTGATG ACGTACGCGC

1501 GAAAGGGCGA CATGTTGCCA TACATCAACC GCGTGGGTAG CTTTGACGTG

1551 GCCTGCACGC GCCACTACGC TGCCGAGCTT CTGCTGGCCT GCGAGCACAT

Primer C

1601 GCACCGCCGT AATGTGGTGC ACCGCGACCT CAAGCCCGAG AACATCCTGC

Primer 4

1651 TCGACGAGGA CATGCACACG CTAATCGCCG ACTTCGGTTC CGCCAAGGTG

1701 ATGACAGCCC ACGAAAGGGC TCTGGCCACG GAGCACTGTT CGGAGCAGCG

1751 GCGCAGCAAC TCCGATGAAG ACGATGAAGA CAGCGACCGC CTGGAAAACG

1801 AAGACGAAGA CTTCTACGAT CGCGATTCGG AGGAGTTGGA CGACCGCGAC

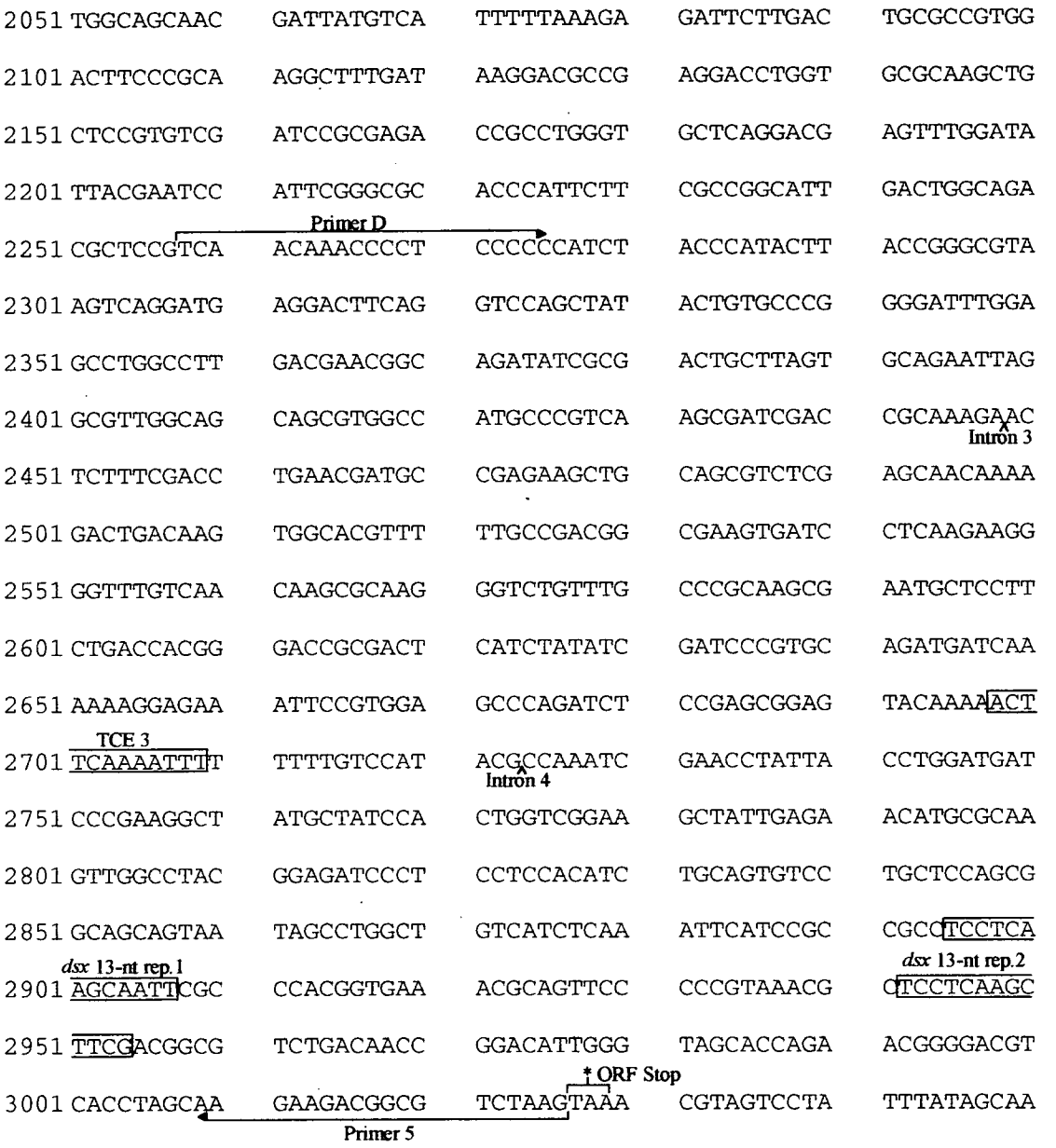
1851 GACGAACAGC AGCAGGAAGA GATGGACTCC CCACGCCATC GTCAAAGGCG

1901 TTACAACCGT CACCGAAAGG CAAGCTTTGT GGGCACTGCC CAGTACGTTT

1951 CACCGGAAGT GCTCCAAAAT GGACCTATAA CCCCGGCGGC GGACCTGTGG

2001 GCACTGGGAT GTATAGTTTA TCAGATGATC GCCGGCCTAC CGCCATTCCG

(continued...)


2051 TGGCAGCAAC GATTATGTCA TTTTTAAAGA GATTCTTGAC TGCGCCGTGG
2101 ACTTCCCGCA AGGCTTTGAT AAGGACGCCG AGGACCTGGT GCGCAAGCTG
2151 CTCCGTGTCG ATCCGCGAGA CCGCCTGGGT GCTCAGGACG AGTTTGGATA
2201 TTACGAATCC ATTCGGGCGC ACCCATTCTT CGCCGGCATT GACTGGCAGA
Primer D
2251 CGCTCCGTCA ACAAACCCCT CCCCCCATCT ACCCATACTT ACCGGGCGTA
2301 AGTCAGGATG AGGACTTCAG GTCCAGCTAT ACTGTGCCCG GGGATTTGGA
2351 GCCTGGCCTT GACGAACGGC AGATATCGCG ACTGCTTAGT GCAGAATTAG
2401 GCGTTGGCAG CAGCGTGGCC ATGCCCGTCA AGCGATCGAC CGCAAAGAAC
Intron 3
2451 TCTTTCGACC TGAACGATGC CGAGAAGCTG CAGCGTCTCG AGCAACAAAA
2501 GACTGACAAG TGGCACGTTT TTGCCGACGG CGAAGTGATC CTCAAGAAGG
2551 GGTTTGTCAA CAAGCGCAAG GGTCTGTTTG CCCGCAAGCG AATGCTCCTT
2601 CTGACCACGG GACCGCGACT CATCTATATC GATCCCGTGC AGATGATCAA
2651 AAAAGGAGAA ATTCCGTGGA GCCCAGATCT CCGAGCGGAG TACAAAAACT
TCE 3
2701 TCAAAATTTT TTTTGTCCAT ACGCCAAATC GAACCTATTA CCTGGATGAT
Intron 4
2751 CCCGAAGGCT ATGCTATCCA CTGGTCGGAA GCTATTGAGA ACATGCGCAA
2801 GTTGGCCTAC GGAGATCCCT CCTCCACATC TGCAGTGTCC TGCTCCAGCG
2851 GCAGCAGTAA TAGCCTGGCT GTCATCTCAA ATTCATCCGC CGCCTCCTCA
dsx 13-nt rep.1
dsx 13-nt rep.2
2901 AGCAATTCGC CCACGGTGAA ACGCAGTTCC CCCGTAAACG CTCCTCAAGC
2951 TTCGACGGCG TCTGACAACC GGACATTGGG TAGCACCAGA ACGGGGACGT
* ORF Stop
3001 CACCTAGCAA GAAGACGGCG TCTAAGTAAA CGTAGTCCTA TTTATAGCAA
Primer 5

(continued...)

3051 TGTGAAATTA ATTTAGTTGA AATTTAGTGC AAACGAAGCG ATGGCGTAGA

3101 AGAGGGCGGG AATAGAAGTA AGCTTAGAAG TAGAAGTAAG TGATGAAGGG

3151 GAAATAATGA TCGTGTCTCT AGTGCTAATT AGAACCAAAT TTCTGTTTTC

Primer E

3201 CGATTTGTAT TGTGCTTAGG GCGAAAATAT AACACTAGAA TAAACTTATC

Primer F PolyA consensus

3251 TAAAGTAAAA TACATCTATA ACGACTAAGT AAATTCAATT TAAAATTTAA

cDNAa 3' end

3301 ATTAAATATT TATATACAGC ATACATAAAT ACAAATATAT TCGTATTTAG

3351 CGCGTAGTTA GTCTCAATTG AAATGAATTA CACACGGAGG CAAGGTGGCA

Primer G

3401 TTGTAGGCTT AAATTAAAAG TAATTTCGTG AAGCAAACTA AAATCCGTAT

3451 TTAAATGGTA TAATACTAGG AAATTATCTG GAAACTGTAA TATACATAAT

3501 ATGTGTATGT ACGTCGGAGA AATAATTTTT GTTTCTGTTA TCGTGGGAAG

3551 ATCACATATA TGTTAATATA AATTTTGTAA ACAGTGTAAC TATGTACAGA

3601 ACTATGTGAG CTATATGCAC ACGGCAAAAA GACCCTAAGT CGTGGGGAAG

3651 GAGATGGGAA TGAGCCGGAA AACCATCGAT GTCACAAAAT CGGTGTTTTT

3701 CCAGCTTCAC GCTCAACTGT TTAGGTAATT CTAATATACA GTTTAACGCT

3751 AATTATCATT ATTTATCATT CTCATTATGC TTTTCGTTTA CCTATTTGCC

3801 CTATAATGAA ATGTATAAGA CTTGACTTGA GCTACCCTAA TAAAAGAACG

3851 AATTTTAAAT TATATTTTTA TTTTGTAAGG GCTACCACTA AAAACTCAAC

3901 AGTCGTTCAA AGATTGTCAA GATTGGCGGT CGTTGTCTCT CCAACTGACT

3951 CCAATTGAAT GTGTGTAAAA CAAGTCTTAT ACAAAGATAT ATGTAATAGA

4001 TGTAAGACAG ACTGCAAAGT TTCTTGAAAC AAATTAGTTA AATGTTGTAA

4051 ATGTCTAAGT TGCTACCACT GCTACTACTA GAACGCGCCC ATCCCCTCCC

4101 ATATTTACCT TTAAGCTACA GTAATTAAGT GATGAGCAGA GGTGTAGAGT

4151 AAGGAGTCGA AGTTGAAAGA AAGCAAAAAT ACATTTTGTA CATACATATC

4201 GCCGTTGGTC GATATACATC TAAACATAAT TTAATTTTTT GTTACTTTTA

Primer 6

4251 AGTAAATTAT TTTTGGCGAC CTAAGTAAAT TAAAATTAAC GCAATATGCA

4301 ACAACACACA TAAGTATACA ATTAATTAAA TTAAACGAAA ATTACGATTT

4351 ATTAAACGAT GTCATAGAAC GGAGAGCCAC TGATAGTGAG AGAAATCTGT

4401 AAAATAAATA TTTAAACAAA TTATATATTA TCTATATATG AGTACCAGAA

4451 CAAGAATGTC AAAAAAGAGA AGCATGTAAA AATAAAAAAT ATATTACGAG

PolyA consensus Primer 7

4501 TAAAACAAAA AAAAAAAAA

The sizes of the cDNAs a and 11 suggest they may represent the male-specific and female carcass-specific transcripts seen on the Northern in figure 3.5. To investigate this possibility, probes were generated to the extended 5' and 3' ends specific to cDNA11. This was carried out via PCR analysis, using primers A and 1 at the 5' end, and primers F and 7 at the 3' end (figure 3.8 for primer positions). The PCR reactions were run on an agarose gel and the single bands produced were excised from the gel and purified. This DNA was radiolabelled by random priming and used to probe Northern blots. The results of this analysis are shown in figure 3.9.

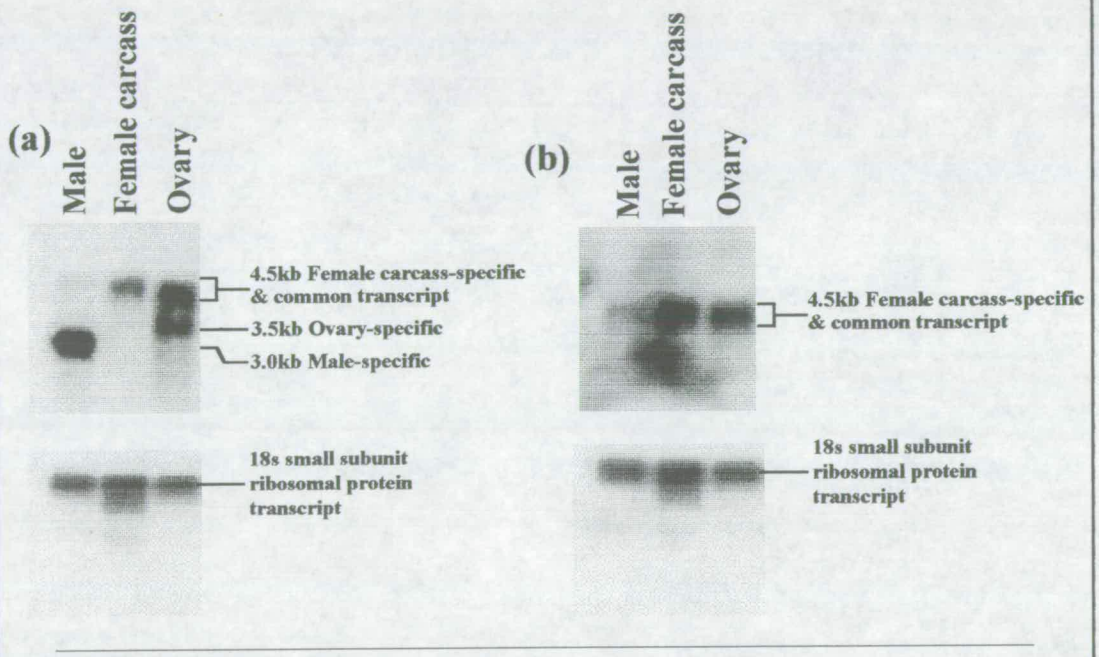


Figure 3.9
Autoradiographs from Northern blots prepared using hand-dissected OrR tissues. Approximately 5υg of polyA$^+$ RNA was loaded per lane. The same filter was used for both (a) and (b). The loading of RNA on this filter was checked by hybridisation with a probe synthesised from an 18s small subunit ribosomal protein cDNA. The female carcass lane appears to have a slightly higher loading relative to the other two lanes. (a) Autoradiograph of the Northern filter hybridised with a probe synthesised from cDNAb. The male-specific, female carcass-specific, ovary-specific and common transcripts all hybridise with this probe. (b) Autoradiograph of the Northern filter hybridised with a probe synthesised from a PCR product containing only cDNA11-specific 3' UTR sequence. Only the female carcass-specific and common transcripts hybridise with this probe.

Interpretation of these results is complicated by the presence of a common transcript on these blots. This band runs either just above or directly on top of the female carcass-specific transcript. The size difference between the common transcript and the female carcass-specific transcript can be clearly seen on the Northern blot shown in figure 3.10. This common band was not seen on earlier Northern blots (figure 3.5) but is apparent on later blots, especially those using polyA$^+$ RNA rather than total RNA, probably due to increased sensitivity. It is possible that this common band represents a partially processed version of the female carcass-specific transcript, and will be discussed in detail in chapter 4. On some gels, the 4.5kb female carcass-specific and common bands run at the same position, possibly due to differences in length of polyA tails, or to small amounts of degradation of the RNA. This would make any size difference between them so small as to be indistinguishable on agarose gel systems. We have been unable to develop a gel system capable of consistently differentiating between these two transcripts.

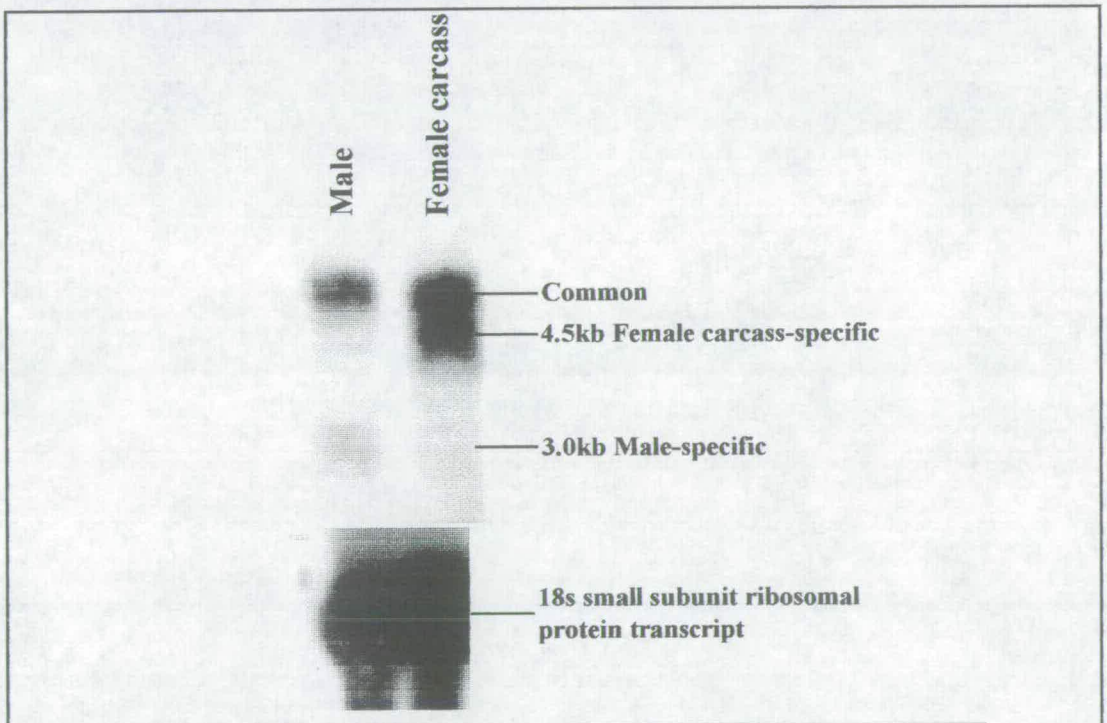


Figure 3.10
Autoradiograph of a Northern blot prepared from OrR tissues. Approximately 20ug of total RNA was loaded per lane. The filter was hybridised with a probe synthesised from cDNAb. The Male-specific, Female carcass-specific and common transcripts all hybridise with this probe. The size difference between the female carcass-specific and common transcripts can be clearly seen. These two transcripts appear to differ in size by 200-300bp. As a loading check, the filter was hybridised with a probe synthesised from an 18s small subunit ribosomal protein cDNA.

Although the female carcass-specific transcript cannot be clearly differentiated in figure 3.9, an increase in intensity is seen. Since the loading check bands have been quantitated and are of virtually identical intensity, it is reasonable to assume that the increase in intensity in the female carcass track is due to the presence of the female carcass-specific transcript.

The 5' Untranslated Region (5' UTR) probe produced no signal on Northern blots (data not shown). Since this probe was much smaller that the 3' UTR probe, this may be explained by small amounts of degradation at the 5' ends of the mRNAs which could reduce the target area for the probe to levels which mean that the signal is too weak to be visualised by this method.

It is quite clear that the PCR probe homologous to the cDNA11-specific 3' UTR hybridises to the female carcass-specific and common transcripts, but not to the male-specific or ovary-specific transcripts. Sequence evidence presented in chapter 4, together with the observed size of the transcripts, makes it unlikely that cDNAa represents the ovary-specific transcript. The fact that the cDNA11-specific 3' UTR probe only hybridises with the female carcass-specific and common transcripts, together with their observed size, shows that this cDNA must represent one of these two transcripts. Again, sequence evidence presented in chapter 4 suggests that cDNA11 represents the female carcass-specific transcript. Thus, we can be fairly certain that cDNAa and cDNA11 represent the male-specific and female carcass-specific transcripts, respectively.

3.2.5 ISOLATION AND MAPPING OF FURTHER GENOMIC DNA

It is clear from figures 3.4 and 3.7 that the existing genomic DNA included in $\lambda fs(1)^{4.3}$ does not contain either the extreme 5' or 3' ends of the isolated cDNAs. In order to determine the structure of the gene it was necessary to isolate genomic DNA representing the entire transcription unit. To this end, restriction mapping of the previously isolated genomic DNA 'phage recombinants, Fix1-Fix7, was carried out.

The recombinant 'phage Fix1-Fix7 were digested with a variety of restriction enzymes which release insert DNA from the 'phage λ arms, and the enzyme *Xba*I was found to produce the most conveniently sized fragments. *Xba*I digests of all 5 'phage are shown in figure 3.11.

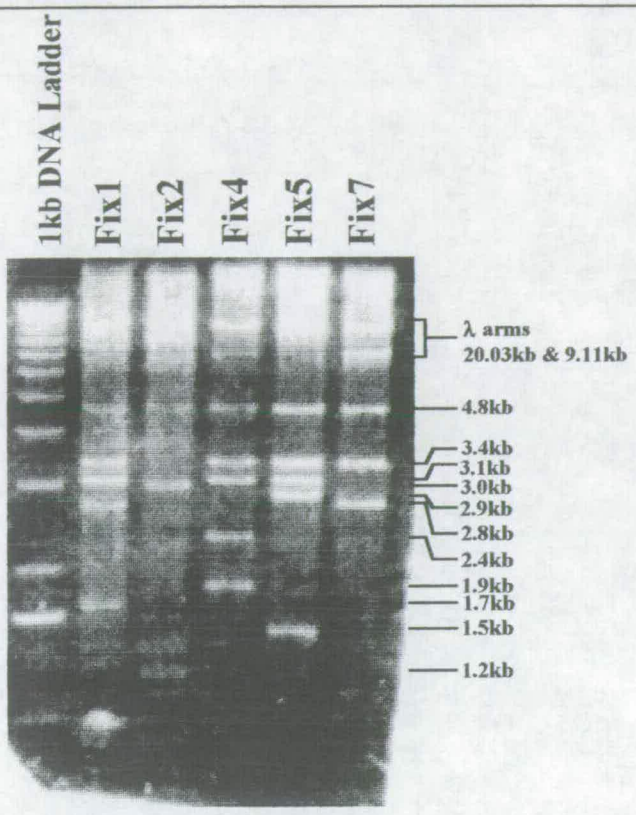


Figure 3.11
1.0% agarose gel showing *Xba*I restriction digests of recombinant genomic DNA bacteriophage Fix1-Fix7. Fragment sizes are indicated in kilobases. See text for details.

All 5 recombinant 'phage contain large genomic DNA inserts, ranging in size from 9kb to 15.8kb. The 'phage λ "FixII" library vector contains a T7 RNA polymerase promoter adjacent to its multiple cloning site which enables the synthesis of RNA across the vector/insert boundary and into the insert itself. This system was applied to generate digoxygenin-labelled RNA probes using the Fix1 recombinant as a template for the transcription reaction. By allowing the reaction to proceed for different times, probes of different lengths were produced and used to probe a Southern blot of a Fix1 *Xba*I digest. Following hybridisation, the probe was visualised using the Lumigen-PPD system (Materials & Methods). The results of this

experiment are shown in figure 3.12. The shortest RNA probe hybridises only with the 2.8kb fragment of Fix1, showing that this fragment must be the one closest to the T7 promoter, at one extreme end of the insert.

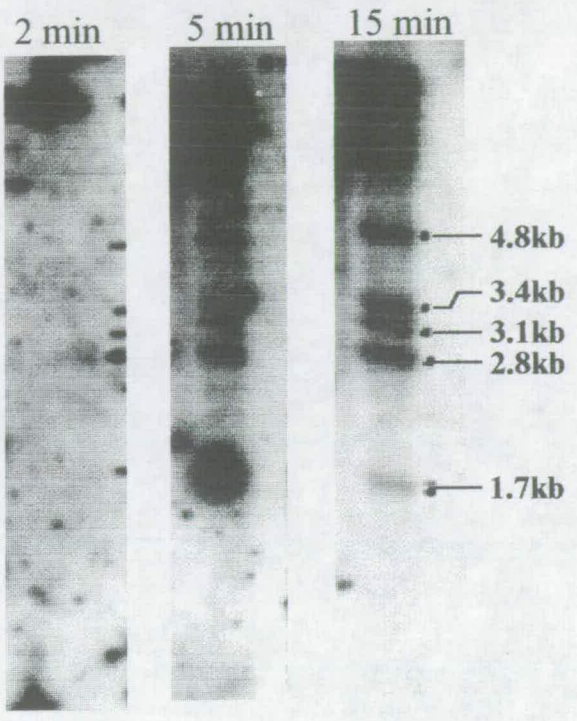


Figure 3.12
Autofluorographs of Southern blots prepared from *Xba*I digests of genomic bacteriophage λ recombinant Fix1. A single 1.0% agarose gel was loaded with three identical Fix1 *Xba*I digests and blotted to a single filter. This filter was then cut into three sections and each section hybridised with a separate digoxygenin-labelled RNA probe. RNA probes were synthesised using Fix1 as a template for the transcription. The transcription reactions were stopped at either 2mins, 5mins and 15 mins to produce RNA probes extending different lengths into the Fix1 insert. Hybridised probe was visualised using the Lumigen-PPD fluorescent system.

Only the 2.8kb fragment of Fix1 hybridises with the 2 min-riboprobe, showing that this fragment must lie at one end of the Fix1 insert. Unfortunately, the autofluorograph from the filter hybridised with the 5 min-riboprobe has a strong background signal obscuring the 1.7kb fragment. However, the result from the 15min-riboprobe shows that the 1.7kb fragment is the least intense of the five bands which suggests that the 1.7kb fragment lies at the opposite end of the Fix1 insert from the 2.8kb fragment.

The gel shown in figure 3.11 was Southern blotted and probed with various segments of DNA to further elucidate the orientation of the fragments in Fix1-7. The following DNA was used to make probes; the Fix2 4.8kb *Xba*I fragment, a cDNAa EcoRV

fragment consisting of the extreme 5' 480bp of the cDNA, the truncated cDNAb comprising the extreme 3' 1.5kb of cDNAa, λfs(1)[4.3] and the Fix5 2.9kb *Xba*I fragment. The results of this are shown in figure 3.13, and table 3.1 shows a summary of which probes hybridised to which *Xba*I fragments of Fix1-7. This data enables the construction of the restriction maps of the recombinants Fix1-7 as shown in figure 3.14.

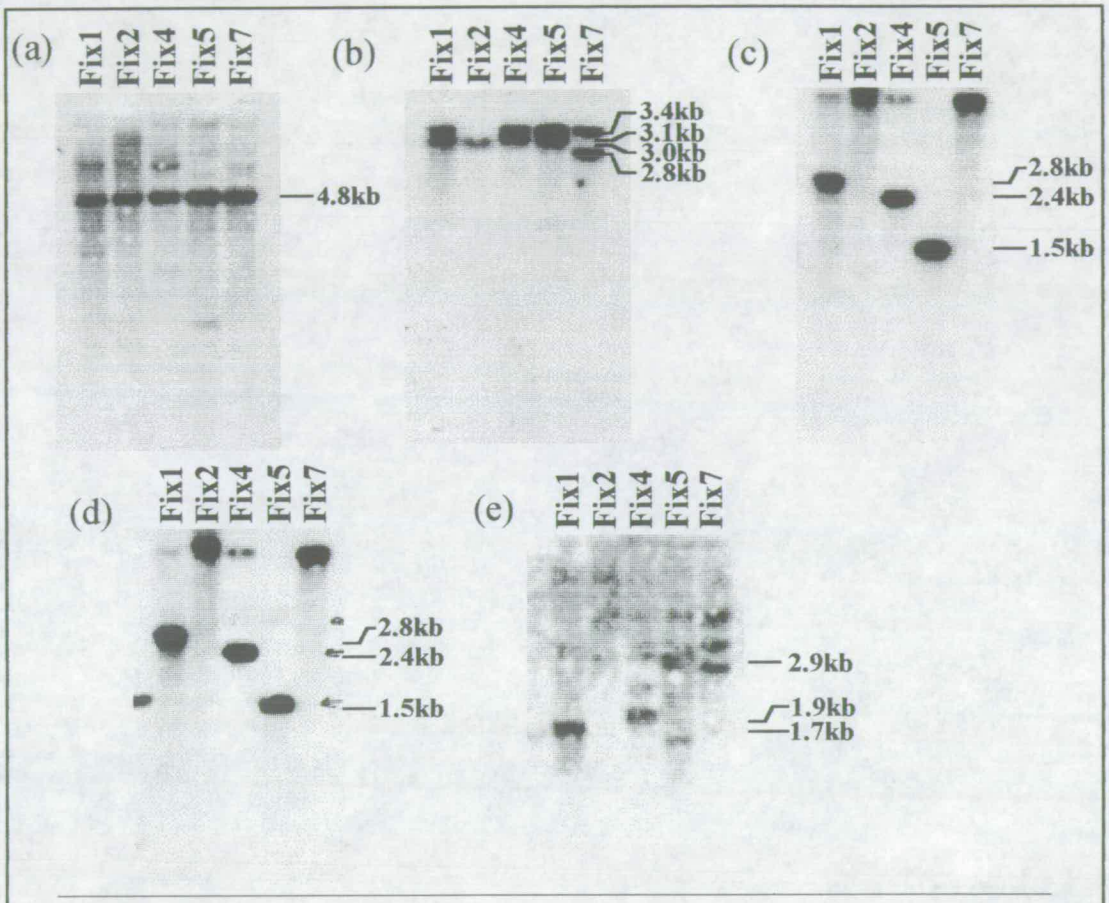


Figure 3.13
Autoradiographs of a Southern blot filter from the agarose gel of Fix1-7 *Xba*I restriction digests, shown in figure 3.11. The sizes of fragments which hybridised with the various probes are indicated in kilobases. These results are summarised in table 3.1. (a) Probe synthesised from the 4.8kb fragment of Fix2. The 4.8kb fragments of all five Fix recombinants hybridise with this probe. (b) Probe synthesised from the 480bp EcoRV restriction fragment of cDNAa, representing the extreme 5' end of the cDNA. The 3.4kb and 3.1kb fragments of Fix1, 4 & 5 hybridise with this probe, as do the 3.4kb and 2.8kb fragments of Fix7 and the 3.0kb fragment of Fix2. (c) Probe synthesised from the truncated cDNAb, representing the extreme 3' 1558bp of cDNAa. This probe hybridises with the 2.8kb fragment of Fix1, the 2.4kb fragment of Fix4 and the 1.5kb fragment of Fix5. (d) Probe synthesised from λfs(1)[4.3]. This probe hybridises with the 2.8kb fragment of Fix1, the 2.4kb fragment of Fix4 and the 1.5kb fragment of Fix5. (e) Probe synthesised from the 2.9kb fragment of Fix5. Despite the high background signal seen here, significant hybridisation is seen to the 1.7kb fragment of Fix1, the 1.9kb fragment of Fix4 and the 2.9kb fragment of Fix5.

	'PHAGE:	Fix1	Fix2	Fix4	Fix5	Fix7
PROBE						
Fix2 4.8kb		4.8kb	4.8kb	4.8kb	4.8kb	4.8kb
cDNAa 5' 480bp		3.1kb 3.4kb	3.0kb	3.1kb 3.4kb	3.1kb 3.4kb	2.8kb 3.4kb
cDNAa 3' 1.5kb		2.8kb	NONE	2.4kb	1.5kb	NONE
$\lambda fs(1)^{4.3}$		2.8kb	NONE	2.4kb	1.5kb	NONE
Fix5 2.9kb		1.7kb	NONE	1.9kb	2.9kb	NONE

Table 3.1
Summary of results shown in figure 3.13. Fragments of genomic DNA bacteriophage λ recombinants, Fix1-7, which hybridise with various probes are shown.

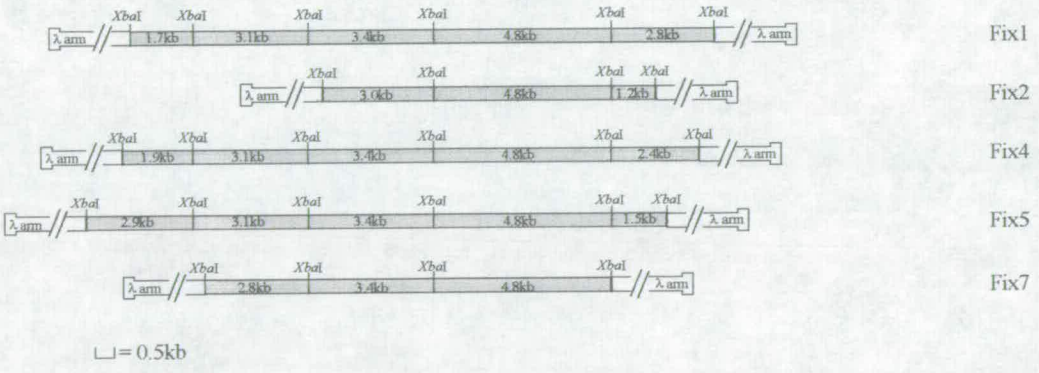


Figure 3.14
Diagram showing restriction maps of bacteriophage λ FixII genomic DNA recombinants, Fix1-Fix7. *Xba*I sites and restriction fragment sizes are indicated.

The following reasoning was used to construct these maps. It is clear that the 4.8kb fragment of all 5 recombinants is in fact the same fragment. We can also say that the 3.1kb fragment of Fix1, 4 and 5 is the same fragment, as is the 3.4kb fragment of Fix1, 4, 5 and 7. Considering Fix1,4 and 5 initially; the data shows that the 3.1kb, 3.4kb and 4.8kb fragments are identical in all 3 recombinants. It follows that the remaining two fragments of each recombinant must be at the extreme ends of the inserts, as they are of different sizes in each recombinant. It has already been shown that the 2.8kb fragment of Fix1 lies at one end of the insert. The fact that the cDNAa 3' probe hybridises with the 2.8kb fragment of Fix1, the 2.4kb fragment of Fix4 and the 1.5kb fragment of Fix5 confirms that all these fragments lie at one end of their inserts. It follows that the 1.7kb, 1.9kb and 2.9kb fragments of Fix1, Fix4 and Fix5 respectively should all lie at the other end of their inserts. This is confirmed by the fact that all 3 fragments hybridise with the 2.9kb fragment of Fix5. With respect to Fix2, we can see that the 3.0kb fragment must lie at the 5' end of the 4.8kb fragment, for if it lay at the 3' end it would have hybridised to the cDNAa 3' probe. The fragment which does lie at the 3' end of the 4.8kb fragment of Fix2 (1.2kb) is apparently too small to reach the sequence covered by the cDNAa 3' probe. This leaves the orientation of the two remaining fragments (3.1kb and 3.4kb of Fix1, 4 and 5; 2.8kb and 3.4kb of Fix7) to be determined. All of these fragments hybridise to the cDNAa 5' probe. This means that the 3.4kb fragment of Fix7 must be the same as the 3.4kb fragments of Fix1, 4 and 5. The 2.8kb fragment of Fix7 must be a truncation of the 3.1kb fragment seen in the other 3 recombinants and as such must lie at the end of the insert. This means that the 3.1kb and 3.4kb fragments of Fix 1, 4, and 5 must lie in the order 3.1kb/3.4kb with the 3.1kb fragment being the most 5'.

The map indicates that the Fix1-7 genomic DNA is highly likely to contain the 5' end of the transcription unit, as shown by the hybridisation pattern of the cDNAa 5' 480bp probe. However, since the probe representing the 3' 1.5kb of cDNAa hybridises with the 1.5kb fragment of Fix5 but not with the 1.2kb fragment of Fix2, the 5' end of the DNA used to make the probe must lie between the 3' ends of these two Fix *Xba*I fragments. Since we know that cDNA11 extends a further 2.7kb 3' of

this point, the Fix1-7 recombinants do not contain enough 3' genomic DNA to cover the 3' end of the transcription unit.

To recover genomic DNA including the 3' end of the transcription unit, the 'phage λ FixII genomic library was screened using a probe made from the PCR product representing the cDNA11-specific 3' UTR. Four recombinant 'phage were isolated which hybridised with this probe. These were designated Genλ1-Genλ4. DNA was prepared from these 'phage, digested with *Xba*I and run out on an agarose gel, as shown in figure 3.15.

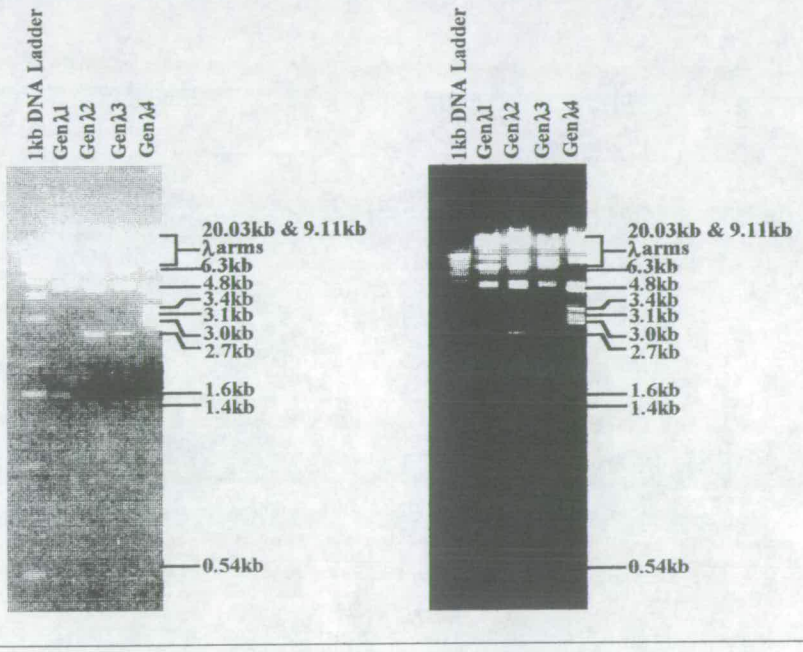


Figure 3.15
Two representations of the same 1.0% agarose gel showing *Xba*I restrictions digests of the genomic DNA bacteriophage recombinants Genλ1-Genλ4. The 1.4kb and 0.54kb fragments are not clearly seen here but were apparent on the original gel and on subsequent gels (data not shown). Genλ1 contains four *Xba*I insert fragments of sizes 6.3kb, 4.8kb, 1.6kb and 0.54kb. Genλ2 and Genλ3 contain five *Xba*I insert fragments of sizes 6.3kb, 4.8kb, 2.7kb, 1.6kb and 1.4kb. Genλ4 contains five *Xba*I insert fragments of sizes 4.8kb (two fragments), 3.4kb, 3.1kb and 3.0kb.

All four 'phage contain large inserts, varying in size from 13.24kb to 19.1kb. Using the same techniques as described for 'phage Fix1-7, the 'phage Genλ1-4 were restriction mapped. Figure 3.16 shows the restriction maps of all 9 recombinant 'phage inserts, and a summary of which probe DNA hybridised to which *Xba*I restriction fragments of all 9 recombinant 'phage is shown in table 3.2.

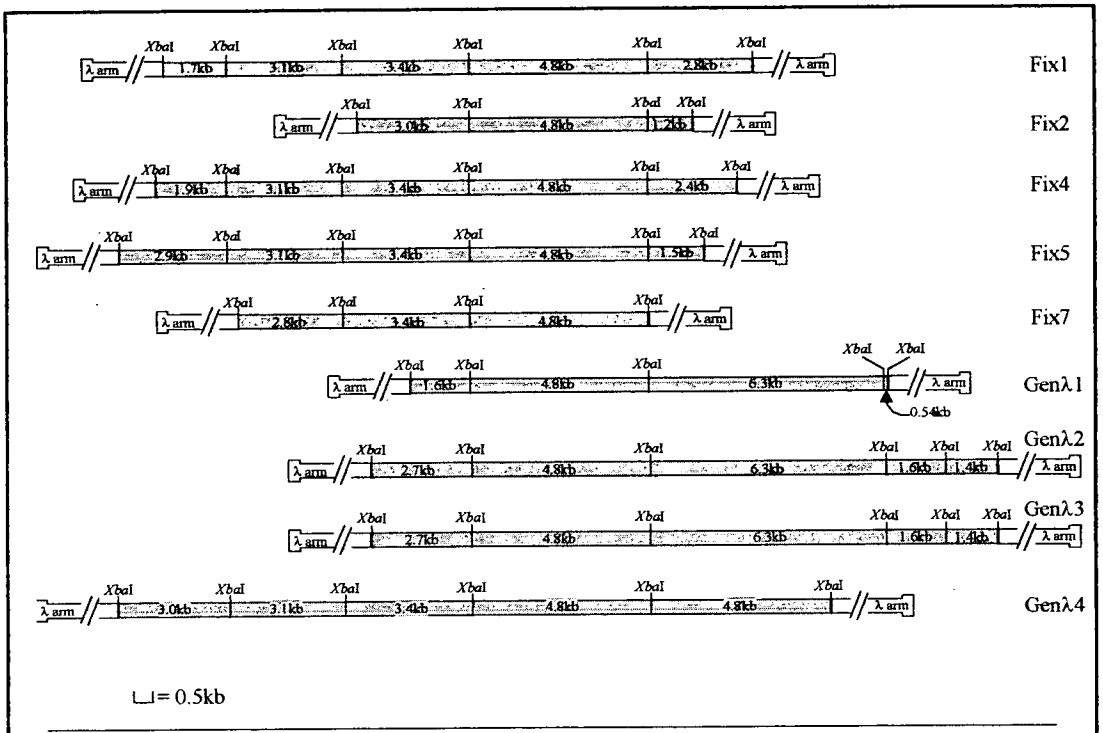


Figure 3.16
Diagram showing restriction maps of all isolated bacteriophage λ FixII genomic DNA recombinants. *Xba*I sites and restriction fragment sizes are indicated.

	'PHAGE:	Fix1	Fix2	Fix4	Fix5	Fix7	Genλ 1	Genλ 2	Genλ 3	Genλ 4
PROBE										
Fix2 4.8kb		4.8kb	4.8kb	4.8kb	4.8kb	4.8kb	4.8kb	4.8kb	4.8kb	4.8kb
cDNAa 5' 480bp		3.1kb 3.4kb	3.0kb	3.1kb 3.4kb	3.1kb 3.4kb	2.8kb 3.4kb	1.6kb	2.7kb	2.7kb	3.1kb 3.4kb
cDNAa 3' 1.5kb		2.8kb	None	2.4kb	1.5kb	None	6.3kb	6.3kb	6.3kb	4.8kb
$\lambda fs(1)^{4.3}$		2.8kb	None	2.4kb	1.5kb	None	N/A	N/A	N/A	N/A
Fix5 2.9kb		1.7kb	None	1.9kb	2.9kb	None	N/A	N/A	N/A	N/A

Table 3.2
Summary of Southern blot results, showing fragments of genomic DNA bacteriophage λ recombinants, which hybridise with various probes.

The Southern hybridisation data makes it highly likely that the entire transcription unit of the gene is contained within the genomic DNA present in the 'phage recombinants Fix1-Genλ4. This cannot be confirmed, however, until the intron/exon structure of the gene is known. The fact that the cDNAa 5' 480bp probe hybridises to the 3.1kb and 3.4kb *Xba*I fragments of the Fix insert DNA (figure 3.13; table 3.1) tells us there must be an intron in this region. This is because there is no *Xba*I site present in the cDNAa fragment from which the probe was made.

3.2.6 ANALYSIS OF GENE STRUCTURE

To determine the precise intron/exon structure of the gene, a differential PCR analysis of cDNA and genomic DNA was carried out. For these PCRs, DNA preps from the 'phage recombinants Fix5 and Genλ2 were used as genomic DNA templates and a DNA prep from the NM1149 'phage recombinant containing the whole of cDNA11 as an insert was used as the cDNA template. PCR reactions were optimised

and carried out as described in Materials and Methods. Loading buffer was added and 1/10th of the entire reaction was run on 1.0% agarose gels. The results of this analysis are shown in figure 3.17, and a summary of the product sizes produced from each reaction is shown in table 3.3.

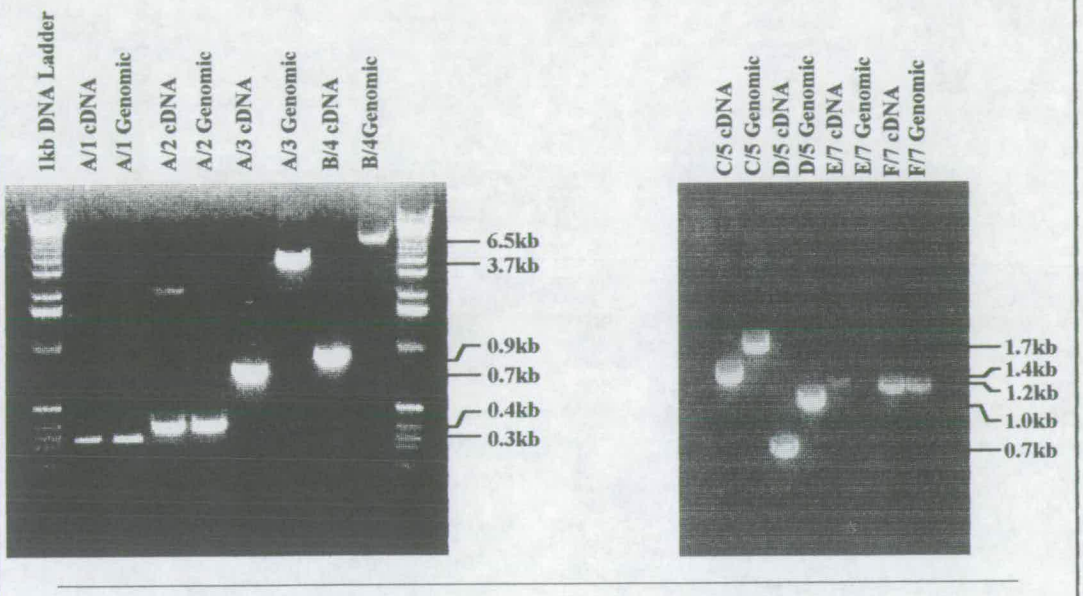


Figure 3.17
1.0% agarose gels showing the PCR products produced from recombinant λ bacteriophage containing either cDNA or genomic DNA inserts. The primers and template used in each reaction is indicated, as are the sizes of products produced. A summary of these results is presented in table 3.3. Three primer sets revealed the presence of intron sequences; 3.0kb between primers A & 3, 5.6kb between primers B & 4 and 0.3kb between primers D & 5. See text for details.

This data suggests the presence of 3 areas from which introns have been spliced out of the transcripts represented by cDNAa and cDNA11. The most 5' region lies between nucleotides +420 and +733 (a distance of 313 nucleotides) of cDNA11, and contains intron sequences of around 3kb in size. This confirms the presence of the putative intron which was suggested by the hybridisation pattern of the 5' 480bp probe shown in figure 3.13. The second lies between nucleotides +787 and +1608 (821 nucleotides) of cDNA11 and contains about 5.6kb of intron sequence. Finally, between nucleotides +2274 and +3010 (736 nucleotides) of cDNA11 there is a further 0.3kb of intron sequence.

PRIMERS USED	TEMPLATE	PRODUCT SIZE	INTRON SIZE
A & 1	cDNA	0.3kb	NONE
	Genomic	0.3kb	
A & 2	cDNA	0.4kb	NONE
	Genomic	0.4kb	
A & 3	cDNA	0.7kb	3.0kb
	Genomic	3.7kb	
B & 4	cDNA	0.9kb	5.6kb
	Genomic	6.5kb	
C & 5	cDNA	1.4kb	0.3kb
	Genomic	1.7kb	
D & 5	cDNA	0.7kb	0.3kb
	Genomic	1.0kb	
E & 7	cDNA	1.2kb	NONE
	Genomic	1.2kb	
F & 7	cDNA	1.2kb	NONE
	Genomic	1.2kb	

Table 3.3
Summary of results of differential PCRs shown in figure 3.17. The positions of the primers used are shown in figure 3.8. Three sets of primers revealed intron sequences; A & 3 (~3kb); B & 4 (~5.6kb); D & 5 (~300bp).

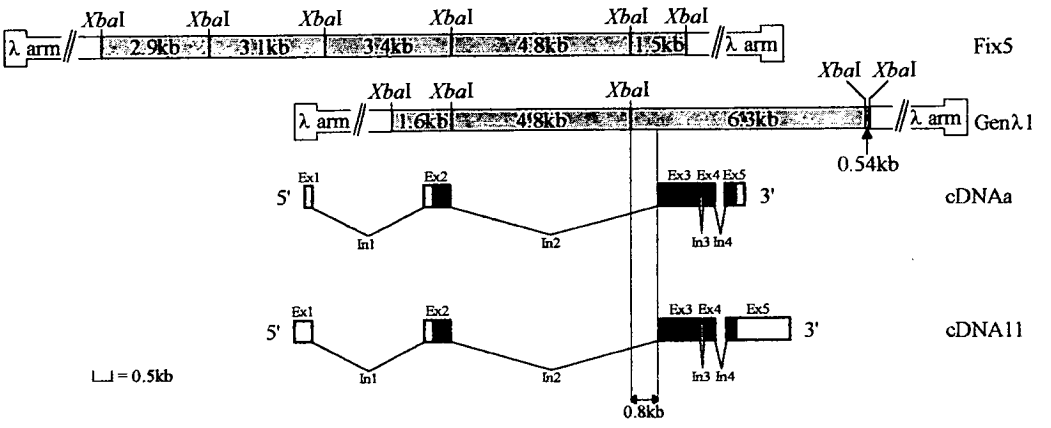
The differential PCR data does not discriminate between single and multiple introns between any two PCR primers. To determine the precise nature of the intron/exon structure of the gene, the genomic DNA-derived PCR products were gel purified and sequenced using dye-labelled cycle sequencing (ABI Prism - see Materials and Methods). The sequence reaction products were analysed on an automated DNA sequencer. This method yielded sequence covering both the 5' and 3' splice site junctions for the introns representing the 3kb and 0.3kb intron sequences, but not for the 5.6kb intron sequences.

The PCR product containing the 5.6kb intron sequences was around 6.5kb in length and it proved difficult to produce enough of this product to yield reliable sequence. However, standard sequencing of the $\lambda fs(1)^{4.3}$ construct reveals the presence of an intron/exon boundary at position +1338/+1339 in cDNA11. The boundary lies between PCR primers B and 4. Thus, if the 5.6kb of intron sequence between these primers is in fact a single intron, then the identified boundary is the 3' end of this intron. We have already seen that the probe made from the 3' 1557bp of cDNAa hybridises to the 1.5kb *Xba*I fragment of Fix5, but not to the 1.2kb fragment of Fix2 (figure 3.13; table 3.1). This places nucleotide position +1713 of cDNA11 between the 3' ends of the 1.5kb and 1.2kb fragments of Fix5 and Fix2, respectively. If we assume that this position lies as far upstream on the genomic DNA as possible (i.e. precisely at the 3' end of the 1.2kb fragment of Fix2), the identified intron/exon boundary lies 375bp upstream of this. Since Southern analysis shows that none of the sequence in cDNA11 hybridises to the 4.8kb *Xba*I restriction fragments of the Fix genomic DNA 'phage recombinants (data not shown), there must be a single intron of at least 4.8kb+[1.2kb-0.375kb]=5.625kb. Thus, it is highly likely that the central 5.6kb of intron sequences revealed by the differential PCR is, in fact, a single intron of at least 5.6kb in size (designated intron 2).

The sequence of the PCR product containing the 3kb of intron sequence shows that this is, in fact, a single intron (designated intron 1), with its intron/exon boundary at position +585/586 in cDNA11. Sequence of the PCR product containing the 0.3kb of intron sequence revealed two introns in this region. The first (intron 3) has its boundary at position +2448/2449 in cDNA11, and is 64bp in size. The final intron (intron 4) is 298bp in size and has its intron/exon boundary at position +2723/2724 in cDNA11. All four introns have donor and acceptor sites which match the consensus to a greater or lesser degree. The relevance of these sites will be discussed in chapter 4.

The relationship between cDNAa, cDNA11 and their corresponding genomic sequence is shown in figure 3.18. This shows that we have now isolated genomic

DNA containing the entire transcription unit which produces the transcripts represented by cDNAa and cDNA11.



	cDNAa	cDNA11
Exon1 (Ex1)	229bp	555bp
Exon2 (Ex2)	754bp	754bp
Exon3 (Ex3)	1111bp	1111bp
Exon4 (Ex4)	276bp	276bp
Exon5 (Ex5)	546bp	1785bp
Intron1 (In1)	~3kb	~3kb
Intron2 (In2)	~5.6kb	~5.6kb
Intron3 (In3)	64bp	64bp
Intron4 (In4)	298bp	298bp

Figure 3.18
Diagram showing relation of cDNAs a & 11 to cloned genomic DNA. The open reading frames of cDNAa and cDNA11 are represented by shaded areas. Actual sizes of all introns and exons are shown. The 5' end of exon 3 must lie at least 0.8kb downstream of the *Xba*I site at the 3' end of the 4.8kb genomic fragment, as indicated.

3.3 DISCUSSION

In this chapter I described the cloning of a gene from *Drosophila melanogaster* which generates three sex-specific transcripts; a 3.0 kb male-specific transcript, a 3.5kb ovary-specific transcript and a 4.5kb female carcass-specific transcript. This gene was cloned via a differential screen designed to isolate genes which give rise to sex-specific carcass transcripts. Therefore, this approach has clearly been successful. The production of sex-specific transcripts implies a sex-specific function and makes this gene a good candidate for a sex differentiation gene. We have provisionally named this gene *serine/threonine kinase 61* (*stk61*), based upon the cytogenetic location and sequence homology of the gene (see Chapter 5), until a name can be assigned upon the basis of phenotype.

Evidence presented in chapter 4 will show that the extended 3' UTR found only in cDNA11, is not present in either the 3.0kb male-specific or 3.5kb ovary-specific transcripts. As well as indicating that cDNA11 does in fact represent the 4.5kb female carcass-specific, this suggests that the 4.5kb female carcass-specific transcript may be subject to 3' UTR-mediated post-transcriptional regulation. The fact that both cDNAs a and 11 (and therefore the male and female transcripts) have exactly the same protein-coding potential, supports the possibility of a role for the extended 3' UTR of cDNA11 (and therefore the female carcass-specific transcript) in modulation of translation or localisation

In *Drosophila*, the part played by the 3' UTR sequences of maternally-supplied oogenic transcripts in spatial localisation has been well documented. The specific localisation of certain mRNAs within the oocyte is required to provide correct protein expression domains, which lead to the determination of embryonic polarity (St Johnston & Nüsslein-Volhard, 1992). Furthermore, 3' UTR sequences have been shown to be sufficient for the correct localisation of *bicoid*, *fs(1)K10*, *nanos*, *oskar* and *orb* maternal mRNAs (Ding & Lipshitz, 1993; Decker & Parker, 1995). Although the system employed by these genes is oocyte-specific, and therefore

unlikely to be directly applicable to the female carcass-specific *stk61* transcript, it does demonstrates how 3' UTR sequences can modulate mRNA localisation, leading to specific protein expression patterns. What may be of more relevance to *stk61* is the observation that the zygotically expressed pair-rule genes; *fushi-tarazu*, *hairy* and *even-skipped* are specifically localised, in a 3' UTR-dependent manner, in the cytoplasm of the cells in which they are expressed (Davis & Ish-Horowicz, 1991). This shows that specific localisation of mRNAs is not limited to the germline in *Drosophila*. It has been proposed that specific localisation of mRNAs to components of the cytoskeleton may result in an increase in translation from those transcripts (Decker & Parker, 1995). This is based on observations that both mRNAs and certain components of the translation machinery are seen to be associated with filementous components of the cytoskeleton. Observations of mRNA localisation in sea urchin eggs have indicated that mRNAs may dissociate from the cytoskeleton when inactive (Moon *et al.*, 1983). Thus, it may be that the 3' UTR in the female carcass-specific transcript of *stk61* is involved in up-regulation of translation by localising this transcript to cytoskeletal components, thereby increasing the local concentration of translation machinery components. A direct demonstration of 3' UTR-mediated translational up-regulation comes from the gene for the human brain-specific amyloid protein (De Sauvage *et al.*, 1992). Two equally stable mRNA species are produced from this gene which are differentially polyadenylated such that one transcript contains an extended 3' UTR. The mRNA with the extended 3' UTR produces more protein than the shorter message and the extended 3' UTR was shown to induce increased translation from a reporter gene-3' UTR fusion. Conversely, specific elements have been identified in the 3' UTRs of the maternally supplied *hunchback* and *bicoid* transcripts which down-regulate translation under the action of Nanos protein (Wharton & Struhl, 1991). These elements have been termed Nanos response elements or NRE's.

Translational repression via the 3' UTR has been demonstrated for the mouse *protamine 1* (*mP1*) gene (Braun *et al.*, 1989). The *mP1* gene is transcribed in the early stages of spermiogenesis but translation does not begin until around 1 week

later, when the spermatids are elongating. Construction of transgenic mice carrying a reporter gene fused to the *mP1* 3' UTR has shown that the 3' UTR of *mP1* directs this translational repression. A testis-specific 18 kDa protein has been shown to be directly responsible for the translational repression of transcripts from another protamine gene, *mP2* (Kwon & Hecht, 1993). This protein is regulated by phosphorylation, being inactivated by dephosphorylation. The mechanism for this repression is not known as yet. However, it is interesting to note that when the *mP1* 3' UTR is replaced by reporter gene sequences in the reporter gene-3' UTR fusion described above, mislocalisation of the transcript was concomitant with onset of translation (Braun *et al.*, 1989). This suggests that the 3'UTRs of *protamine* genes may repress translation by sequestering the mRNA away from areas in the cell which have a high concentration of translation machinery components.

The stability of mRNAs can also be regulated by 3' UTR sequences. For example, the mammalian Transferrin receptor mRNA 3' UTR contains iron response elements (IRE's), acting as target sequences for a *trans*-acting factor which protects the message from endeolytic cleavage (Owen & Kühn, 1987; Müllner & Kühn, 1988; Koeller *et al.*, 1989). A 3' UTR-mediated mRNA-stabilisation has also been proposed for the transcripts of the *exuperantia* gene (Crowley & Hazelrigg, 1995-see chapter 1 for details). However, since we see no consistent difference in the levels of the three *stk61* transcripts, it seems unlikely that the extended 3' UTR of the female carcass-specific transcript acts to either stabilise or destabilise this transcript.

It is possible that the extended 3' UTR of the *stk61* female carcass-specific transcript acts to either promote or repress translation of the message. This may or may not involve specific cytoplasmic relocalisation. In general, translational regulation via 3' UTR sequences appears to be utilised early in development, when little transcription is occurring. Thus, if the function of the extended 3' UTR in the *stk61* female carcass-specific transcript is to modulate translation of STK61 protein, we might expect the female carcass-specific function of *stk61* to include early embryonic components. Preliminary whole mount *in situ* RNA hybridisation evidence (D.

Clyde, pers. comm.) indicates that *stk61* transcript is not expressed in a spatially restricted pattern in 3rd instar larva, with staining seen in the brain and all imaginal discs studied. However, this does not rule out the possibility of intracellular localisation of mRNA, or of specific STK61 protein localisation. It is also possible that hybridisation to the common *stk61* transcript is masking specific localisation of the female carcass-specific transcript. The sex of the larvae used in these RNA *in situ* hybridisation experiments was not known. Further experiments, using sexed larvae, may reveal quantitative differences in signal, in specific tissues, which would indicate the presence of female carcass-specific transcript.

In the next chapter I will discuss evidence concerning further regulation of the *stk61* transcripts, with a view to elucidating the possible role for *stk61* in sex differentiation.

CHAPTER 4

REGULATION OF SEX-SPECIFIC TRANSCRIPTS PRODUCED FROM A NOVEL GENE IN *DROSOPHILA MELANOGASTER*

4.1 INTRODUCTION

Examination of *Drosophila* which carry mutations in genes of the sex determination hierarchy makes it clear that the genes which are responsible for differentiation of the vast majority of sex-specific features are under hierarchy control at some level. In the case of the *yp* genes, this control is direct, with Dsx protein acting as a transcriptional regulator of the genes. No other genes have yet been isolated which are under direct Dsx control. It is also clear that *dsx* is not epistatic to all sex-specific differentiation genes. Certain traits lie downstream of *tra* and *tra-2*, but not *dsx*, such as a pole cell sex-determining somatic signal, certain aspects of behaviour and developmental repression of the male-specific Muscle of Lawrence. Similarly, the overall size of the fly appears to be dependent upon *Sxl*, but not *tra-2* or *dsx*, since XX/*tra-2* flies, while being morphologically male, are of female size. Other than the *yp* genes, no non-gonadal sex differentiation genes have been cloned.

In the previous chapter we described the cloning of a gene which produces a male-specific transcript of 3.0kb and a female carcass-specific transcript of 4.5kb. We showed that this size difference was likely to be brought about by differential ploydenylation resulting in a female-specific 3'UTR. The production of sex-specific transcripts implies a sex-specific function for this gene, making it a good candidate for a sex differentiation gene. If this gene does indeed have a role in sex differentiation, we would expect it to be under the control of at least one of the genes of the sex determination hierarchy. In this chapter, I describe experiments using flies mutant for sex determination hierarchy genes, designed to test this hypothesis.

4.2 RESULTS

4.2.1 THE MALE-SPECIFIC TRANSCRIPT IS TESTIS-SPECIFIC

The production of a male-specific transcript from *stk61* does present something of a paradox, as the gene was originally identified on the basis of preferential hybridisation to female cDNA. However, the sex-specific cDNA used for the initial screen contained no gonad-derived cDNA. If the male-specific transcript were, in fact, gonad-specific, it would account for the fact that the genomic recombinants screened did not show strong hybridisation to male non-gonadal cDNA. To test this hypothesis, gonads were dissected from male flies and PolyA$^+$ RNA prepared from both gonad and carcass tissue. This RNA was used in Northern blot analysis, with a probe synthesised from cDNAa, as shown in figure 4.1.

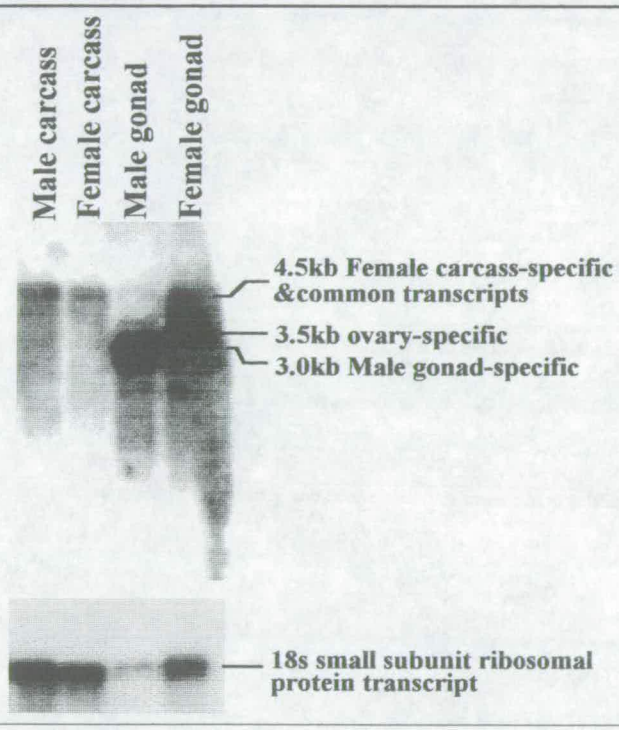


Figure 4.1

Autoradiograph of a Northern blot prepared from hand-dissected OrR tissues. (~ 5υg of polyA$^+$ RNA loaded per lane). The filter was hybridised with a probe synthesised from cDNAa. The 3.0kb male-specific transcript is only seen in RNA from male gonad tissue. The filter was stripped and re-probed with a probe synthesised from an 18s small subunit ribosomal protein cDNA. The male gonad lane appears to be underloaded relative to the other lanes. However, a good signal is produced from this lane using the cDNAa probe. Thus, the low levels of ribosomal protein transcript is probably a consequence of generally low levels of ribosomal protein gene expression in testis tissue.

It is clear from this that the male-specific transcript is entirely gonad-specific. To determine whether the transcript is expressed in testis or paragonial tissue, a similar Northern blot was carried out, but this time using total RNA prepared from accessory gland and testis tissue, as shown in figure 4.2.

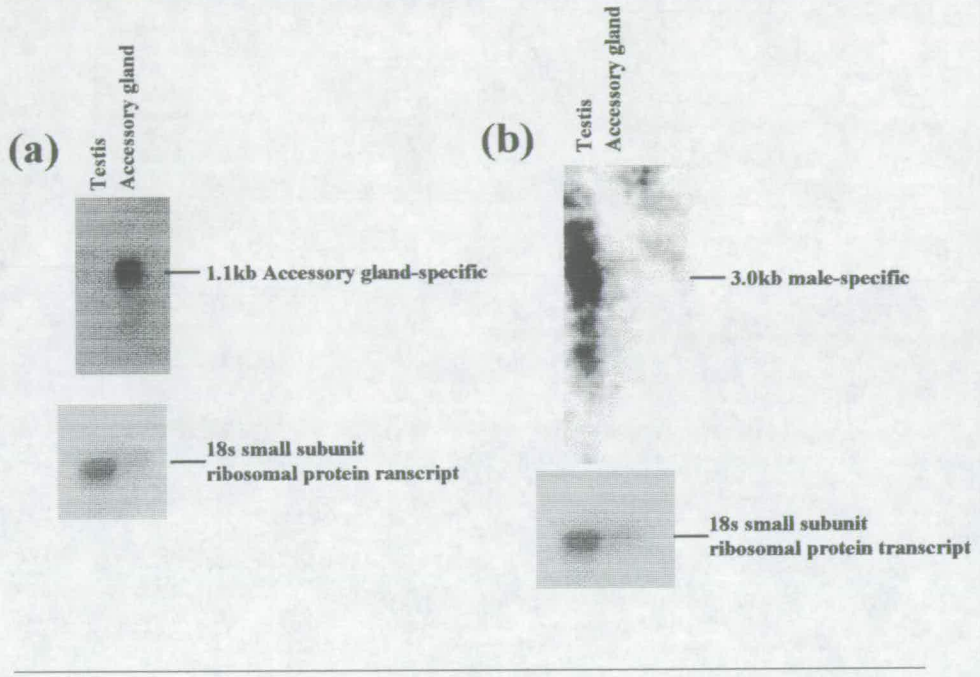


Figure 4.2
Autoradiographs of Northern blots prepared from hand-dissected male adult Oregon R tissue. Approximately 20ug of total RNA was loaded per lane. A probe synthesised from an 18s small subunit ribosomal protein cDNA was used as a loading check. The testis track appears to be underloaded from this. However, ribosomal proteins are generally of low abundance in the testis, and re-probing the same filter with a *PD-10a* cDNA (courtesy M.Wolfner, Cornell University, NY) representing an accessory gland-specific transcript, (a), shows that accessory gland RNA is present in sufficient quantity and quality. When this filter was probed with a probe synthesised from cDNAa, (b), male-specific transcript was only detected in RNA from testis tissue. No signal was seen in RNA from accessory gland tissue.

Male-specific *stk61* transcript was only detected in testis RNA. The accessory gland lane appears to be underloaded compared to the testis lane. However, when the same filter was hybridised with a probe made from a cDNA, *PD-10a*, representing an accessory gland-specific transcript (courtesy M. Wolfner, Cornell University, NY), the correct band was clearly seen (figure 4.2a). The fact that the 3.0kb male gonad-specific transcript is entirely testis-specific has been confirmed by whole

mount *in situ* hybridisation which exhibits signal in testis tissue, but not accessory gland (D. Harbison, pers. comm.). It is clear, therefore, that the 3.0kb male-specific transcript is, in fact, testis-specific. However, this does not show whether the 3.0kb transcript is present in somatic or germline cells. In either case, its production is highly unlikely to be governed by genes of the sex determination hierarchy. Studies on *yp* expression in *tra-2* temperature sensitive mutants show that, although *yp* expression requires *tra-2* in the fat body, *tra-2* is not needed for *yp* expression in the follicle cells of the ovary. Similar results have been obtained for transcripts expressed specifically in the somatic component of the male gonad (see chapter 1 for details). In the case of germline cells, a hierarchy gene-dependent signal is required from the soma to determine a pole cell as female. However, once a pole cell has been determined as male or female, it becomes hierarchy-independent. In the testis, *tra-2* is required for correct spermatogenesis to occur. This spermatogenic function of *tra-2* is independent of *tra*, and is due, at least in part, to a role in the regulation of *exuperantia* (*exu*) transcripts. *exu* is required for correct gametogenesis to occur in both sexes. A set of ovary-specific and testis-specific *exu* transcripts are produced, the ovary transcripts having an intron spliced out of the 3' UTR which remains present in the testis transcripts. It has been shown that *tra-2* has a role in the production of the testis *exu* transcripts, since they are produced less efficiently in *tra-2* mutants. Several 13 nucleotide repeats (13-nt repeats), similar to the repeats present downstream of the *dsx* transcript sex-specific splice site, have been found in and around the intron which is spliced from the *exu* ovary transcripts. In *dsx*, these repeats are thought to act as binding sites for Tra and Tra-2 proteins which then stabilise the splicing apparatus at the female-specific splice acceptor site (see chapter 1 for details). In *exu*, it is thought that Tra-2 may associate with the 13-nt repeats, interfering with the splicing process and inhibiting splicing of the female-specific intron. However, it is not yet known which of the identified 13-nt repeats, if any, play a role in *exu* regulation in the testis. Tra-2 protein is also required in the testis to inhibit the splicing out of the M1 intron from the *tra-2* transcript. *dsx*-like 13-nt repeats have also been identified in *tra-2* transcripts and may play a similar role to that which has been postulated for the repeats in *exu*.

For these reasons, we would not expect the 3.0kb transcript to be under the continual control of genes of the sex determination hierarchy, although there may be some level of Tra-2-mediated regulation, as has been suggested in the cases of the *exu* and *tra-2* transcripts.

4.2.2 *TRA-2* REGULATION OF THE TESTIS-SPECIFIC TRANSCRIPT

Figure 4.3 shows a Northern blot of RNA prepared from *tra-2*^{ts} flies, hybridised with a probe made from cDNAa. In these mutants, *tra-2* is functional at 16°C and produces no active product at 29°C. In wild type flies, the 3.0kb transcript is only present in *XY* (i.e. male=*tra-2* OFF) flies. Figure 4.3 shows that it is also present in *XX/tra-2*^{ts} flies which have been raised at 29°C (*tra-2* OFF), but not present in *XX/tra-2*^{ts} flies raised at 16°C (*tra-2* ON). Although, at first glance, this seems to indicate *tra-2* regulation of the transcript, the appearance of the transcript in *XX/tra-2*^{ts} flies raised at 29°C is more likely to be due to the development of testes in these flies. Indeed, when we look at the gonads of *XX/tra-2*^{ts} flies raised at 29°C (figure 4.3b), we see that testes do develop in these mutants.

XX/tra-2^{ts} flies raised at 29°C develop as pseudomales; which is to say that they are male in all respects apart from their size and the fact that they are sterile, due to lack of the *tra-2*-mediated spermatogenic functions. Since *tra-2* is required for correct germline development in the testis, the fact that the 3.0kb transcript from *stk61* is present in testes from XX and XY *tra-2*^{ts} homozygotes raised at 29°C (both of which lack active Tra-2) argues against, rather than for, any role for *tra-2* in the production of this transcript. If there is no *tra-2* regulation of this transcript, it suggests that the transcript is either involved in some *tra-2*-independent gametogenic function, or is not involved in gametogenesis at all but is actually expressed in the somatic component of the testis. However, since testis-specific *exu* transcripts are also found in testes lacking Tra-2, albeit at reduced levels, it is clear that whatever control *tra-2* exerts over *exu* transcripts, it is not as absolute as the control which *tra* and *tra-2*

exert over the *dsx* transcript in the female fat body. Thus, we cannot rule out a role for *tra-2* in the regulation of 3.0kb *stk61* testis transcript.

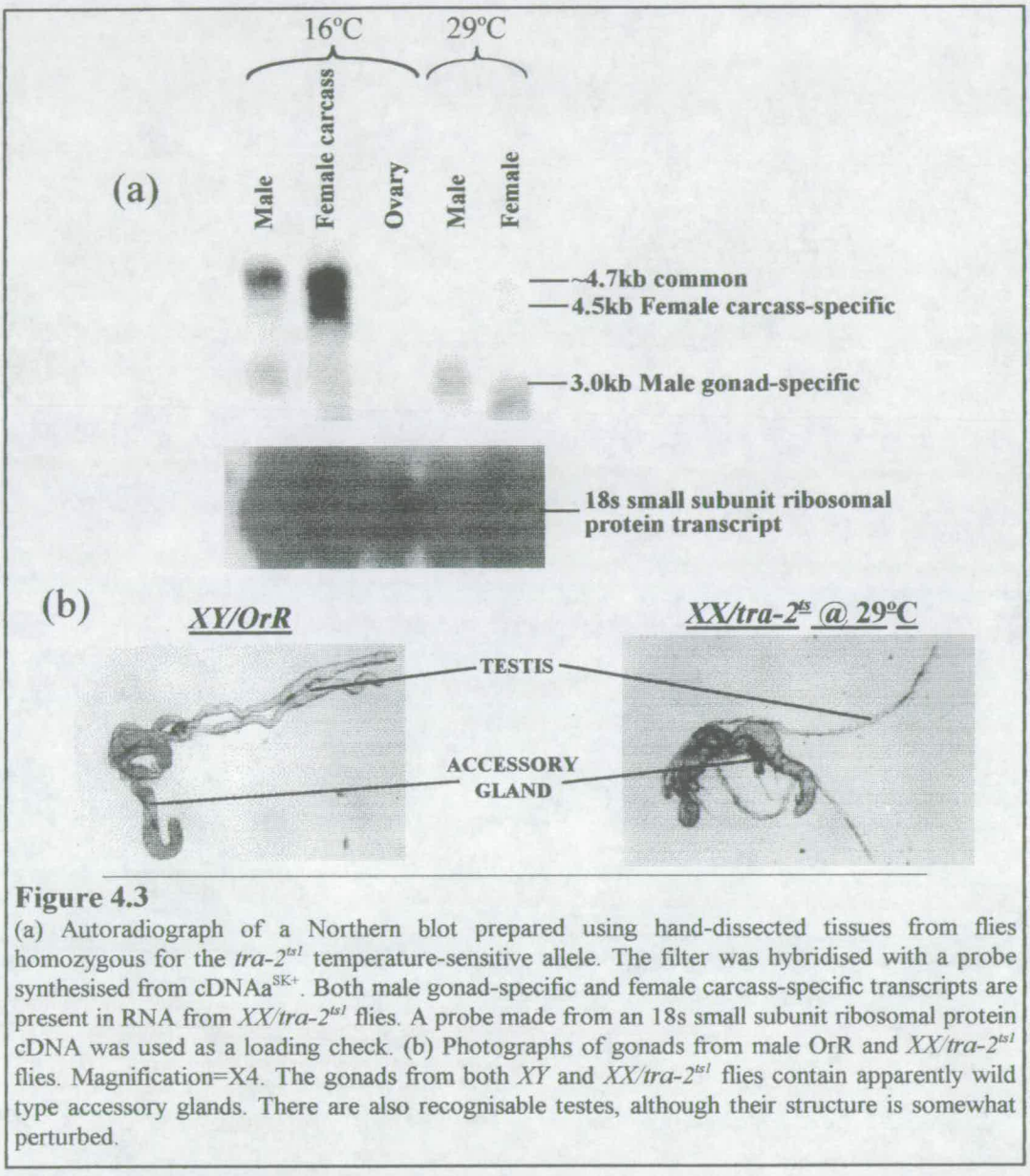


Figure 4.3

(a) Autoradiograph of a Northern blot prepared using hand-dissected tissues from flies homozygous for the *tra-2*^{ts1} temperature-sensitive allele. The filter was hybridised with a probe synthesised from cDNAa^{SK+}. Both male gonad-specific and female carcass-specific transcripts are present in RNA from *XX/tra-2*^{ts1} flies. A probe made from an 18s small subunit ribosomal protein cDNA was used as a loading check. (b) Photographs of gonads from male OrR and *XX/tra-2*^{ts1} flies. Magnification=X4. The gonads from both *XY* and *XX/tra-2*^{ts1} flies contain apparently wild type accessory glands. There are also recognisable testes, although their structure is somewhat perturbed.

4.2.3 *TRA* REGULATION OF THE TESTIS-SPECIFIC TRANSCRIPT

The evidence from the *tra2*^{ts} flies makes it reasonable to suppose that any fly which develops testes will have the 3.0kb *stk61* transcript present. Northern blots using *tra*^{FEM} mutants, which constitutively express the *tra* female-specific cDNA, and *dsx* null mutants show this to be the case.

Figure 4.4 shows a Northern blot analysis of RNA from *tra*^{FEM} mutant flies.

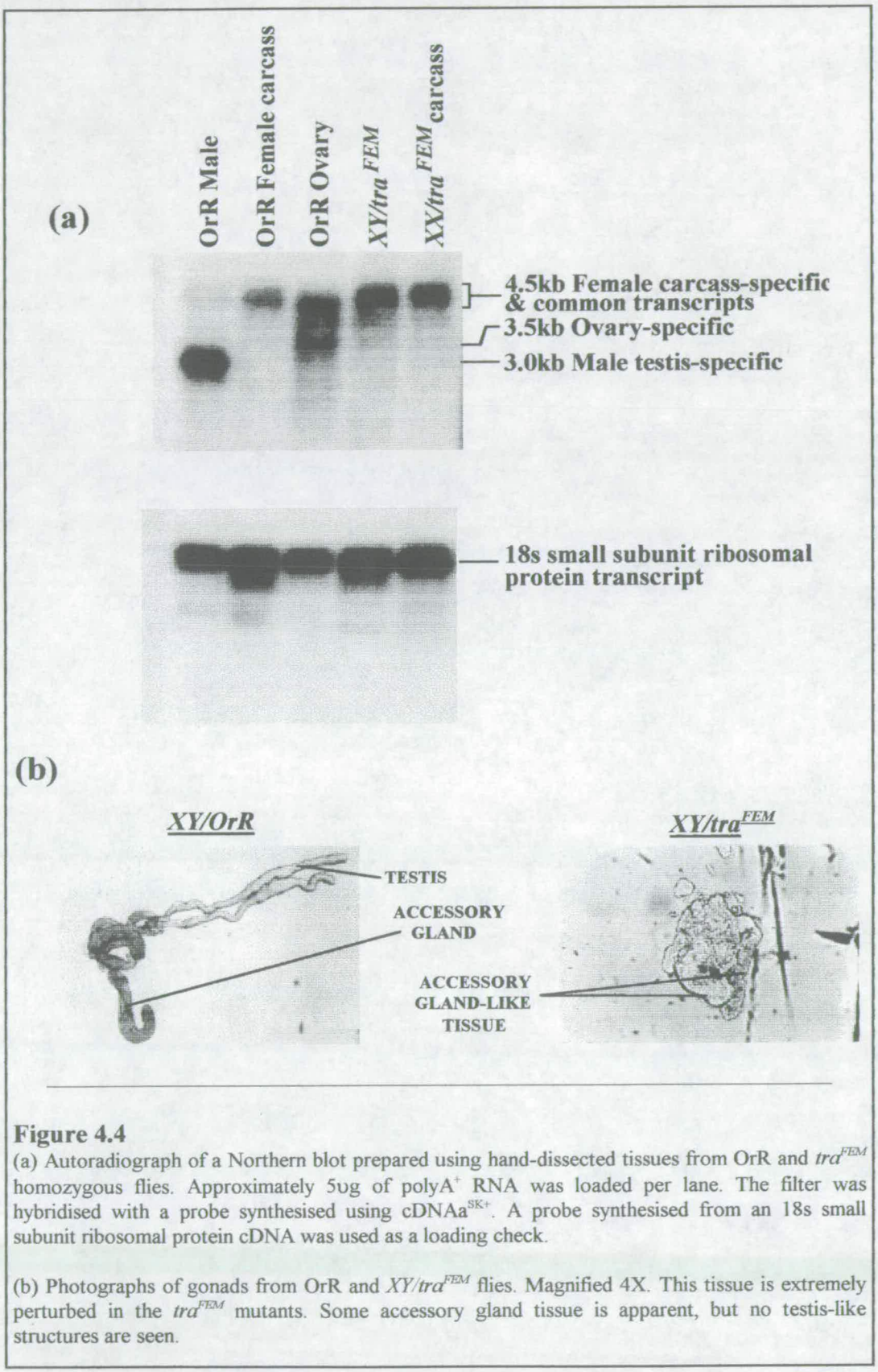


Figure 4.4

(a) Autoradiograph of a Northern blot prepared using hand-dissected tissues from OrR and *tra*^{FEM} homozygous flies. Approximately 5υg of polyA^{+} RNA was loaded per lane. The filter was hybridised with a probe synthesised using cDNAa^{SK+}. A probe synthesised from an 18s small subunit ribosomal protein cDNA was used as a loading check.

(b) Photographs of gonads from OrR and *XY/tra*^{FEM} flies. Magnified 4X. This tissue is extremely perturbed in the *tra*^{FEM} mutants. Some accessory gland tissue is apparent, but no testis-like structures are seen.

Again, the probe was made using cDNAa as a template. Both male and female wild type flies produce active Tra-2. Thus, *XY/tra*^{FEM} flies develop as pseudofemales due to the presence of active *tra* gene product. They appear female in morphology, although they are of wild type male size. Examination of their gonads (figure 4.4b) reveals that they do not develop recognisable testes, while there does appear to be fairly well developed accessory glands. As predicted, the 3.0kb *stk61* testis transcript is not present in these flies.

4.2.4 *DSX* REGULATION OF THE TESTIS-SPECIFIC TRANSCRIPT

The 3.0kb *stk61* testis transcript is not present in *XY/dsx* null mutants, as shown by the Northern blot in figure 4.5. Again, the gonads from these flies do not contain recognisable testes (figure 4.5b). Both *XY/dsx* and *XX/dsx* null mutant flies are intersexual, exhibiting a mixture of both male and female characteristics (Laugé, 1980). The flies are of wild-type size, but sex combs bristles are partially developed in both *XY/dsx* and *XX/dsx* flies (Hannah-Alava & Stern, 1957). The genitalia and analia are composed of both male and female features and male-like abdominal pigmentation is seen in both *XY* and *XX dsx* null mutants. The gonads of both *XX* and *XY dsx* flies contain male-like ducts and accessory glands, although the level of differentiation of these structures can vary from individual to individual. Well developed ovaries can develop in *XX/dsx* flies, but ovarian structures are rarely seen in *XY/dsx* individuals and, when present, are poorly differentiated (Laugé, 1980). The gonads from the *XY/dsx* flies used in the Northern blot experiment shown in figure 4.5 contained no testes, as shown in figure 4.5(b). The 3.0kb *stk61* testis transcript was not seen in the RNA from flies of either *XX/dsx* or *XY/dsx* genotype.

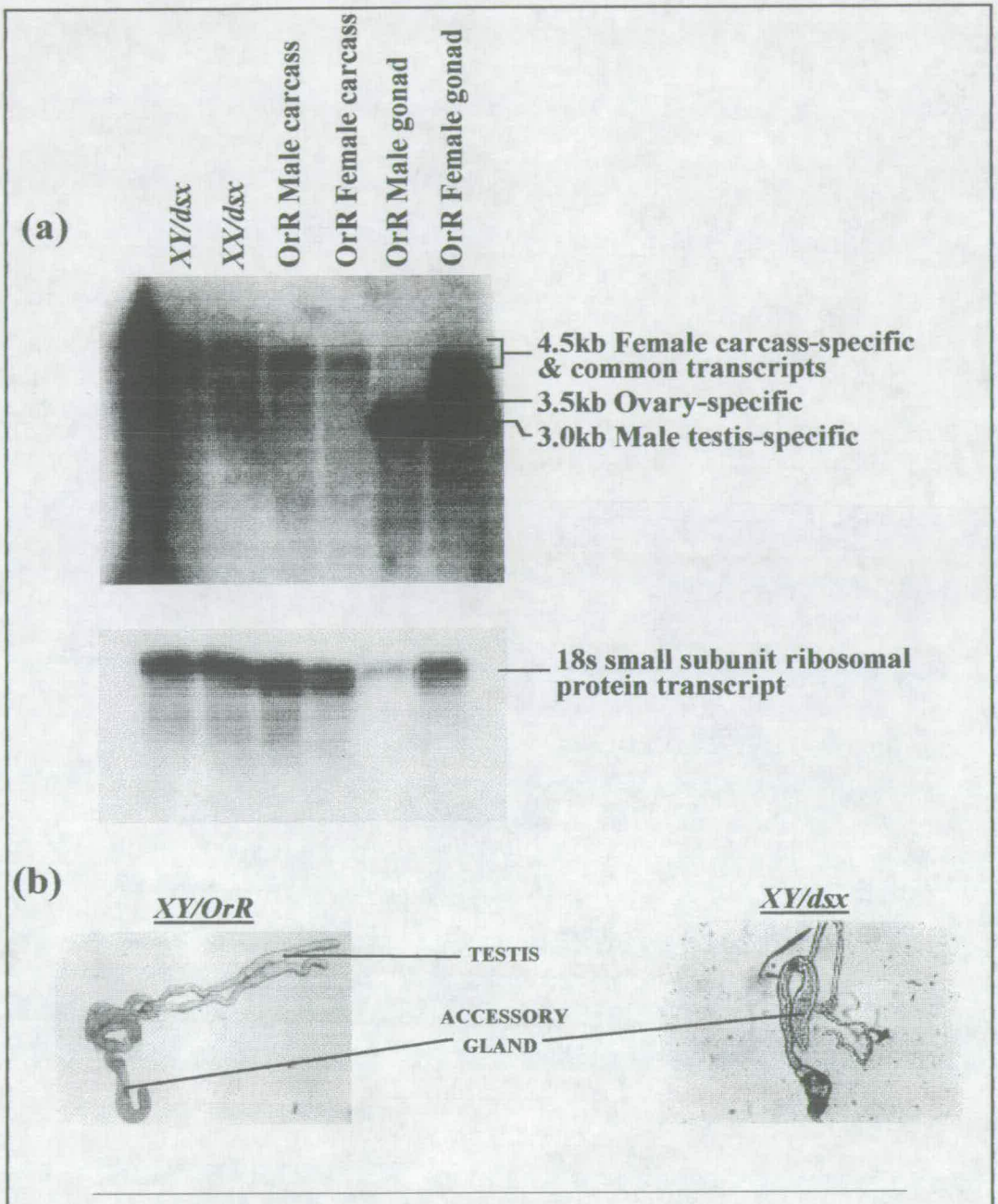


Figure 4.5

(a) Autoradiograph of a Northern blot prepared using hand-dissected tissues from OrR and *dsx* mutant flies. Approximately 5υg of polyA$^+$ RNA was loaded per lane. The filter was hybridised with a probe synthesised using cDNAa^{SK+}. No male gonad-specific transcript is seen in the *dsx* mutant flies. A probe synthesised from an 18s small subunit ribosomal protein cDNA was used as a loading check.

(b) Photographs of gonads from male OrR and *XY/dsx* flies. Magnified 4X. The gonads from the *XY/dsx* flies contain some recognisable accessory gland tissue, but no testis tissue is seen.

4.2.5 THE TESTIS-SPECIFIC TRANSCRIPT CONTAINS TCE ELEMENTS

Examination of the sequence of the cDNA thought to represent the testis transcript (cDNAa) provides some interesting clues as to the possible regulation of this transcript. In the 5' UTR of cDNAa there are two 12 nucleotide repeats which have similarity to an element known to play a role in translational control of testis-specific transcripts. This is known as the translational control element, or TCE (consensus=ACATCNAAATTT; see figure 4.6). In order to understand the relevance of this, it is necessary to discuss some aspects of spermatogenesis which were not covered in Chapter 1.

nt+1
CGAATGTTAA GAATCAAAGC TAAGAATTAA ATAAAATTAA AGCCGCACAC ATACATATAC

ACAGGCACGC GCAGAGTCGA AAAAATACGC GCGCTCGCTC TGTACAGTGT GTGCGTGTAC

nt+173 TCE 1
GCGGTGGCTG GCTCTTCTTC TTCATTCGTG TGCCCATTGG AATTTGTACC CCAAATCGAA

ATTACAACAT AGACAAACAA AGAGCGCCCG TCTGCCCCAG CACCTAAACA TCAACGGTAC

ACAGCAACAG CTCCAATTGC CAGGCAGCGG CGCTAGCGGA ATCGCAGCGG CAGCGTAATC

nt+359
ACGGTAGCAT CGGATTGCGG CGAAAACTGC AGCAGTAACG GCACCGAGCA TCAGCAGCAC

TCE 2 Translation start site
TTCAACATTG CTACCACCAC AGCAACTTCG GCGACAGAGG CAACAATACC GGCTATGGCC

Element	Sequence	Matches
Consensus	ACATCNAAATTT	12/12
TCE 1	AAATCGAAATTA	10/12
TCE 2	ACTTCAACATTG	9/12
TCE 3	ACTTCAAAATTT	11/12

Figure 4.6
Sequence of the 5' end of cDNAa, showing positions of TCE-like elements; TCE 1 & TCE 2. TCE 3 is present between introns 3 & 4 (figure 4.7). The homology of TCE 1, 2 & 3, to the consensus sequence is shown. Nucleotides which are perfect matches to the consensus are highlighted.

Radiolabelled uridine experiments show that virtually no transcription occurs after the primary spermatocyte stage of spermatogenesis (Olivieri & Olivieri, 1965). Thus, the transcripts required for protein synthesis during development of the haploid spermatids into functional spermatozoa (spermiogenesis) are present before

spermiogenesis begins. The *Mst(3)CGP* gene family comprises a set of genes which encode structural sperm tail proteins. These genes are transcribed in the primary spermatocyte (Kuhn *et al.*, 1988). However, antibody *in situ* hybridisation to preparations from testis tissue, shows that translation of transcripts from members of this gene family does not occur until very late in spermiogenesis, when the spermatids are fully elongated (Schäfer *et al.*, 1993). The *Mst(3)CGP* transcripts contain the 12-nt TCE element at an invariant position within the 5' UTR, 28bp downstream from the 5' end. Transgenic flies have been made using fusion constructs consisting of a LacZ reporter gene, incorporating 5' UTR sequence from a member of the *Mst(3)CGP* gene family at its 5' end (Kuhn *et al.*, 1988). LacZ staining of gonads from male 3rd instar larvae (which do not contain spermatogenic cells beyond the primary spermatocyte stage) showed that no translation of LacZ was occurring. When the TCE was deleted from the fusion gene, however, LacZ staining could be seen at these stages. Thus, the TCE element is clearly regulating the onset of translation from the LacZ fusion transcripts. When transversion mutations of bases 5 and 7 (A<–>C) were present in the TCE, similar levels of LacZ expression were seen in 3rd instar gonads as when the TCE is completely deleted. This indicates that the integrity of nucleotides 5 and 7 in the TCE is vital to its function in regulating translation. Transversion of bases 9 and 10 (AT–>GC) also caused some loss of regulation but the effect was not as marked as with bases 5 and 7.

Gel shift experiments using *in vitro* transcript containing a TCE, revealed three bands from which three differently sized proteins could be purified (Kempe *et al.*, 1993). These bands were only produced when the *in vitro* transcript was incubated with extracts from testis tissue. Extracts from any other tissue produced no bands. Only one protein could be purified following UV crosslinking, however, suggesting that a single protein may bind to the TCE which, when bound, attracts other proteins to it. The TCE element is clearly vital to the production of the gel-shifted bands, as only the largest gel shift band was seen when the *in vitro* transcript used contained the TCE carrying the base 5 and 7 transversions. This band was also relatively unstable, as shown by cold competitor controls. Since this mutant TCE also resulted

in loss of translational regulation of the LacZ fusion transcript, the protein binding revealed by the gel shift experiments is likely to be functionally relevant. Disruption of protein interactions with the *in vitro* transcript was also seen when transversions of bases 9 and 10, and base 12 (T–>G) were present in the TCE, but to a lesser degree.

The two TCE-like elements present in the 5' UTR of the 3.0kb *stk61* testis-specific transcript, are good matches to the consensus sequence. The upstream element (TCE 1) matches at 10/12 nucleotides and the downstream element (TCE 2) at 9/12. The mutational analysis of the *Mst(3)CGP* genes showed that nucleotides 5 and 7 were vital for TCE function. Both of the elements present in *stk61* have these two nucleotides conserved. Nucleotides 9, 10 and 12 were also shown to affect TCE function when mutant, although less dramatically than positions 5 and 7. Both of the TCE-like elements in *stk61* match the consensus at positions 9 and 10 but differ at position 12, with the upstream element having an A rather then a T and the downstream element exhibiting a G rather than a T. Although transversion at base 12 was shown to affect protein complex formation in the gel shift experiments described above (middle gel shift band did not form) the complexes which did form were quite stable, indicating that this base does not play as vital a role in TCE function as bases 5 and 7.

Another factor which is vital for the correct functioning of the *Mst(3)CGP* TCE elements is the position of the TCE element relative to the 5' end of the transcript. In all of the transcripts studied from this gene family, the TCE is invariably positioned 28bp from the 5' end. If the element is repositioned 54bp downstream of the 5' end, all TCE-dependent translational control is lost. In cDNAa, the TCE like elements lie 173bp and 359bp from the 5' end of the cDNA. A similar arrangement to this exists in another gene, *Mst59D* (Huang & Nöthiger, pers. comm. in Schäfer *et al.*, 1995). The 5' UTR of the *Mst59D* transcript contains two TCE-like elements, also matching the consensus at 9/12 and 10/12 positions. This "TCE box" of *Mst59D* is located 60bp from the 5' end of the transcript, but the position of the box does not appear to

be vital to its translational control function, provided the overall integrity of the TCE elements within it is maintained. It seems likely, therefore, that the TCE elements present in the 5' UTR of the 3.0kb *stk61* testis transcript represent a "TCE box" of the kind seen in *Mst59D*, regulating the translation of this transcript as has been described for the *Mst(3)CGP* gene family. It may be that the position-independent TCE box, containing two TCE elements, represents a separate class of TCE regulation from that seen in transcripts containing single, position-dependent, TCE elements. One model to account for this could be that a single TCE is unable to prevent translation once it has initiated at the 5' end of the transcript. Thus, it must be placed close to the 5' end to ensure that translation cannot initiate. However, if two TCE elements are present in tandem, they may be capable of "derailing" the translational apparatus as it proceeds down the transcript in search of the translational start site. This would account for the fact that the TCE box present in *Mst59D* is still operative even when repositioned, while the single TCE elements found in transcripts from the *Mst(3)CGP* gene family must remain at the extreme 5' end of the transcript to exert translational control.

The onset of translation of the transcripts from the *Mst(3)CGP* genes is associated with a secondary polyadenylation event which lengthens their polyA tails from 140 to 380 nucleotides (Schäfer *et al.*, 1990). This polyadenylation event is also controlled by the TCE sequence. In the mutational analysis described above, this secondary polyadenylation was not seen either when the TCE was deleted, or when nucleotides 5 and 6 were mutated. This is interesting with regard to the 3.0kb testis transcript from *stk61*. The cDNA thought to represent this transcript, cDNAa, is polyadenylated 1.2kb upstream from the site used to yield cDNA11, the cDNA thought to represent the 4.5kb female carcass-specific transcript. The secondary polyadenylation event observed for the *Mst(3)CGP* gene transcripts is an extension of the existing tail, rather than the utilisation of a second polyadenylation site. However, it may be that the utilisation of the upstream polyadenylation site in cDNAa occurs as part of a secondary polyadenylation under the control of the TCE sequences in the transcript. This could explain why the upstream polyadenylation

site is not used in the female carcass-specific transcript as shown by sequence analysis of cDNA11. The observation that gel shifts of TCE-containing transcripts only occur with testis extracts, indicates that the trans-acting factors which act at the TCE are testis-specific. Thus, if polyadenylation at the upstream site, as in cDNAa, is part of a TCE-mediated process, we would not expect to see the upstream site used in carcass-specific transcripts.

These considerations make it highly likely that the 3.0kb *stk61* testis transcript is expressed in the germline, playing some part in spermiogenesis.

4.2.6 REGULATION OF THE FEMALE CARCASS-SPECIFIC TRANSCRIPT

The initial objective of the differential screen, by which *stk61* was isolated, was to identify putative sex differentiation genes. We might expect any non-gonadal sex-specific transcripts from such genes to be under the continual control of the genes of the sex determination hierarchy, as is the case for the *yp* genes. To investigate whether *stk61* is under such control, we used Northern blots to examine the presence of the non-gonadal sex-specific 4.5kb transcript.

This analysis proved problematic due to difficulties connected with visualising the 4.5kb transcript on Northern blots. Firstly, this transcript is of very low abundance, necessitating the production of blots which were both low in background signal and of very high sensitivity. This was complicated by the low viability of some of the mutant strains used, making large scale isolation of polyA$^+$ RNA impractical. However, the biggest problem was the presence of the common transcript which runs just above the 4.5kb transcript (see figure 3.10), and is present in RNA isolated from carcass and gonads of both sexes. On the blot shown in figure 3.10, a size difference of several hundred base pairs is seen between the 4.5kb transcript and the common transcript. However, in most cases, the common transcript was found to run so close to the 4.5kb transcript as to be inseparable on agarose gels.

4.2.6.1 The female carcass-specific transcript contains *dsx*-like 13-nt repeats.

The presence of the *stk61* common transcript presents theoretical as well as practical problems. If cDNA11, which contains full protein-coding potential, represents the common transcript, then the sex-specific *stk61* transcripts would apparently be redundant. For this reason, it is more probable that cDNA11 represents the female carcass-specific transcript and that the common transcript is, in fact, a partially processed RNA. Where size differences between the 4.5kb and common transcripts have been observed, the common transcript appears to be large enough to contain one or both of the smaller introns, intron 3 (64bp) and intron 4 (298bp), which are spliced out of the protein coding region of cDNAs a and 11. Thus, the common transcript may be identical to the transcript represented by cDNA11 but still containing intron 3 and/or intron 4 in the protein coding sequence, which would prevent active protein from being translated. Transcripts are not seen which are large enough to contain introns 1 and 2. However, these species would be far too large to be visualised using standard RNA preparation and Northern blotting methods.

Since work on the *yp* genes indicates that sex-specific gene expression in non-gonadal tissue is most likely to be under continual hierarchy gene control, we were particularly interested in looking at the regulation of the carcass-specific transcripts of *stk61*. Figure 3.10 shows that male carcass tissue contains only the common transcript, while female carcass tissue contains the 4.5kb female carcass-specific transcript and the common transcript. As we have suggested, the female carcass transcript may in fact be a fully processed form of the common transcript, differing only in the splicing of intron 3 and/or intron 4. The question which arises is; why would these introns not be spliced out of the common transcript in male carcass tissue?

This situation is similar to that discussed in chapter 1 for the *dsx* transcript. In the case of *dsx*, a suboptimal splice acceptor site at the 3' end of the sex-specific intron is stabilised by Tra and Tra-2 proteins so that this site is only used in females. Tra and

Tra-2 act at a series of six tridecamer repeats (13-nt repeats) downstream of the splice acceptor site. Examination of the *stk61* sequence surrounding introns 3 and 4 provides some interesting clues as to what may be happening to regulate the expression of the *stk61* female carcass-specific transcript.

Two sites with homology to the *dsx* 13-nt repeats are present downstream of *stk61* intron 4, lying at positions +2895 (*dsx* 13-nt rep 1) and +2942 (*dsx* 13-nt rep 2), as shown in figure 4.7. Also shown in this figure is a third TCE-like element, the relevance of which will be discussed later in this chapter.

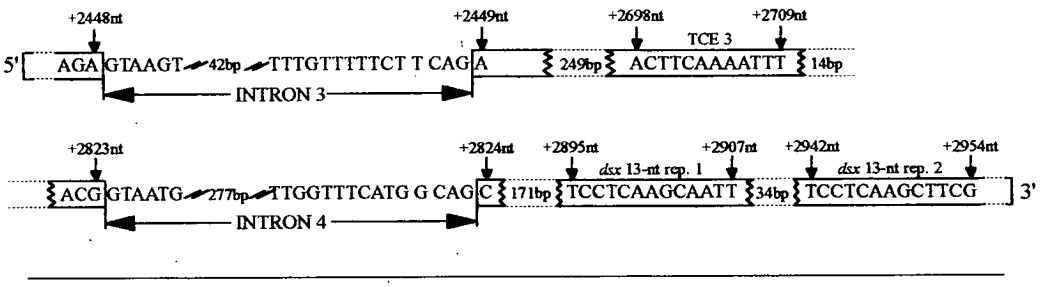


Figure 4.7
Sequence of relevant consensus sequences around introns 3 & 4 of cDNAs a & 11. Nucleotide positions are given relative to cDNA11. See text for details.

The upstream *dsx*-like 13-nt repeat matches the *dsx* consensus at 9/13 positions and the downstream repeat at 8/13 positions. The *dsx*-like 13-nt repeats found in the *exu* gene exhibit similar levels of homology to the *dsx* 13-nt repeats. What is also interesting, is the similarity which the two *stk61* repeats bear to each other, with the first 9 nucleotides of both repeats being identical. This strongly suggests that these two repeats are, in fact, functionally relevant. The consensus sequence for a splice acceptor site has been identified as Y_{11}NCAG, where Y is a pyrimidine base and N is T, C, G or A (Ohshima & Gotoh, 1987). In *dsx*, the female-specific splice acceptor

site contains only 6/11 pyrimidine bases in the Y_{11}, or polypyrimidine (polyY), tract (Burtis & Baker, 1989). The other, non Tra/Tra-2-regulated, acceptor sites have better matches to the consensus, containing 8-10 Y's in their polyY runs. Introduction of an 18nt polypyrimidine tract into the *dsx* female-specific splice acceptor site enables this site to be efficiently utilised, even in the absence of Tra and Tra-2 (Hoshijima *et al.*, 1991). Therefore, in *dsx*, the reason why the female-specific splice site is not used in the absence of Tra and Tra-2 is that it lacks a good polyY tract, making it a poor substrate for the splicing machinery. The sequences of the intron donor and acceptor sites within *stk61* are shown in table 4.1.

	Donor	Acceptor	Y-tract Ycontent
Consensus	(C/A)AG/GTAAGT	YYYYYYYYYYY N CAG/(G/A)	11
Intron 1	AAC/GTAAGT	CATATATTTTT C CAG/A	8
Intron 2	XXX/XXXXXX	ATCTTCTCCTT G CAG/T	10
Intron 3	AGA/GTAAGT	TTTGTTTTTCT T CAG/A	10
Intron 4	ACG/GTAAGT	TTGGTTTCATG G CAG/C	7

Table 4.1
Donor and acceptor splice sites from introns 1-4 of cDNAs a & 11. Nucleotides which are perfect matches to the consensus are highlighted. The number of pyrimidine nucleotides within the polypyrimidine tract of the splice acceptor sites are shown. Y=pyrimidine; N=T, C, G or A; X=nucleotide at this position not known.

The acceptor site which is the poorest match with the consensus sequence is that of the most downstream intron, intron 4. As well as having the lowest overall number of Y's in the polyY run, it lacks a consecutive run of Y's longer than 4. As we can see from figure 4.7, the two *dsx*-like 13-nt repeats lie 172bp downstream of this intron. In *dsx*, the most upstream of the 13-nt repeats lies 295bp downstream of the regulated splice site. Thus, the two *stk61 dsx*-like 13-nt repeats are ideally positioned to be involved in the regulation of the intron 4 acceptor site. The intron 3 acceptor site is a very good match to the consensus, having 10/11 Y's in the polyY run and so is unlikely to require any additional regulation to be efficiently utilised. Thus, if the common transcript is a partially spliced form of the female carcass-specific transcript, it is most likely to contain only intron 4.

The Northern in figure 3.10 shows that the common transcript from *stk61* is still present, even in female carcass tissue. If the common transcript is indeed a partially spliced form of the female carcass-specific transcript, then we can see that splicing rarely occurs to completion. The Northern in figure 4.8 has been hybridised with a probe made from a *dsx* cDNA. This probe hybridises to both male and female-specific *dsx* transcripts. We can see here that, in the female, none of the transcript remains in the unspliced (i.e. male) form. The smaller 2.9kb male-specific band is a minor product of the *dsx* gene in males, and is thought to be generated by use of a second polyadenylation site located upstream of that used in the 3.9kb male-specific transcript (Burtis & Baker, 1989). Hence, this minor product is not relevant to this discussion.

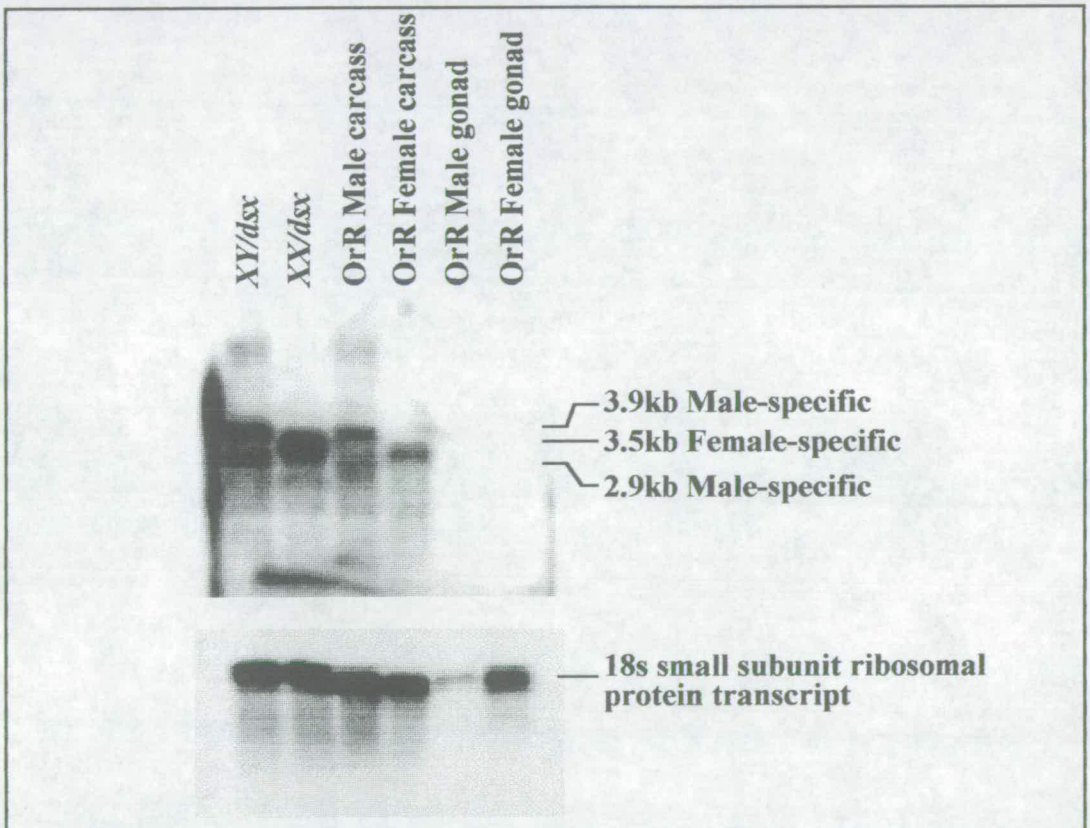


Figure 4.8
Autoradiograph of a Northern blot prepared using hand-dissected tissues from OrR and *dsx* mutant flies. Approximately 5υg of polyA$^+$ RNA was loaded per lane. The filter was hybridised with a probe synthesised using a *dsx* cDNA. Both mutant and wild type flies produce the expected male-specific and female-specific transcripts. After a longer exposure, both transcripts can also be seen at greatly reduced levels in the gonads. In the female, splicing at the female-specific acceptor site occurs to completion since only the 3.5kb transcript is seen in RNA from these flies. A probe synthesised from an 18s small subunit ribosomal protein cDNA was used as a loading check.

If Tra and Tra-2 are acting on transcripts from *stk61*, as they do in *dsx*, then why is it that common transcript (putatively unspliced form) is still present in female tissue? It is interesting to note that if all but two of the 13-nt repeats are deleted from the *dsx* transcript, a significant drop in the utilisation of the the female-specific splice site is observed (Hoshijima *et al.*, 1991). This might explain why the *stk61* common transcript is still seen on Northern blots, in female carcass tissue. Another possibility is that competition between the acceptor sites of introns 3 and 4 for components of the splicing apparatus reduces the efficiency of intron 4 splicing. The two introns are only separated by 374bp, and the intron 3 acceptor site is a vastly more optimal site than that of intron 4 (figure 4.7; table 4.1). Interestingly, in *dsx*, when the female-specific acceptor site is deleted, several downstream cryptic acceptor sites start to become utilised (Ryner & Baker, 1991). In transcripts from the wild type gene, these cryptic sites are never used. This is presumably due to the fact that the female-specific site is a better match to the consensus sequence and efficiently competes with the cryptic sites for splicing apparatus components (in the presence of Tra and Tra-2). Thus, a model where the efficacy of *stk61* intron 4 splicing is hindered by the superior efficiency of intron 3 splicing, is clearly a strong possibility. It may be that the combination of a sub-optimal polyY run and a the competition with intron 3, makes the splicing of intron 4 a very inefficient process, such that the aid of Tra and Tra-2, acting at two downstream 13-nt repeats, is required to enable any fully spliced transcript to be produced.

4.2.6.2 *tra-2* regulation of the female carcass-specific transcript.

In an attempt to determine whether *tra-2* is involved in production of the *stk61* 4.5kb female carcass-specific transcript, a Northern blot was performed using RNA extracted from *tra-2*^{ts} mutant flies (figure 4.3). If, as we suggest, the common transcript is spliced under the control of Tra/Tra-2 proteins, to produce the 4.5kb female carcass-specific transcript, we would not expect the 4.5kb transcript to be produced in the absence of Tra-2. However, we do see a very small amount of 4.5kb transcript in RNA prepared from carcasses of *XX/tra-2*^{ts} flies raised at 29°C (*tra-2*

inactive). This result does not rule out the possibility of *tra-2* involvement, since temperature sensitive mutations are more likely to be hypomorphic rather than completely amorphic. Thus, there may be enough active *tra-2* transcript present at the restrictive temperature to enable some splicing to occur. If this is the case, we might expect to be able to detect *yp* transcripts in *XX/tra-2*^{ts} female carcass tissue at the restrictive temperature. However, when the same filter was re-probed with a *yp* probe, no *yp* transcript was detected from the carcass tissue of 29°C *XX/tra-2*^{ts} flies (figure 4.9).

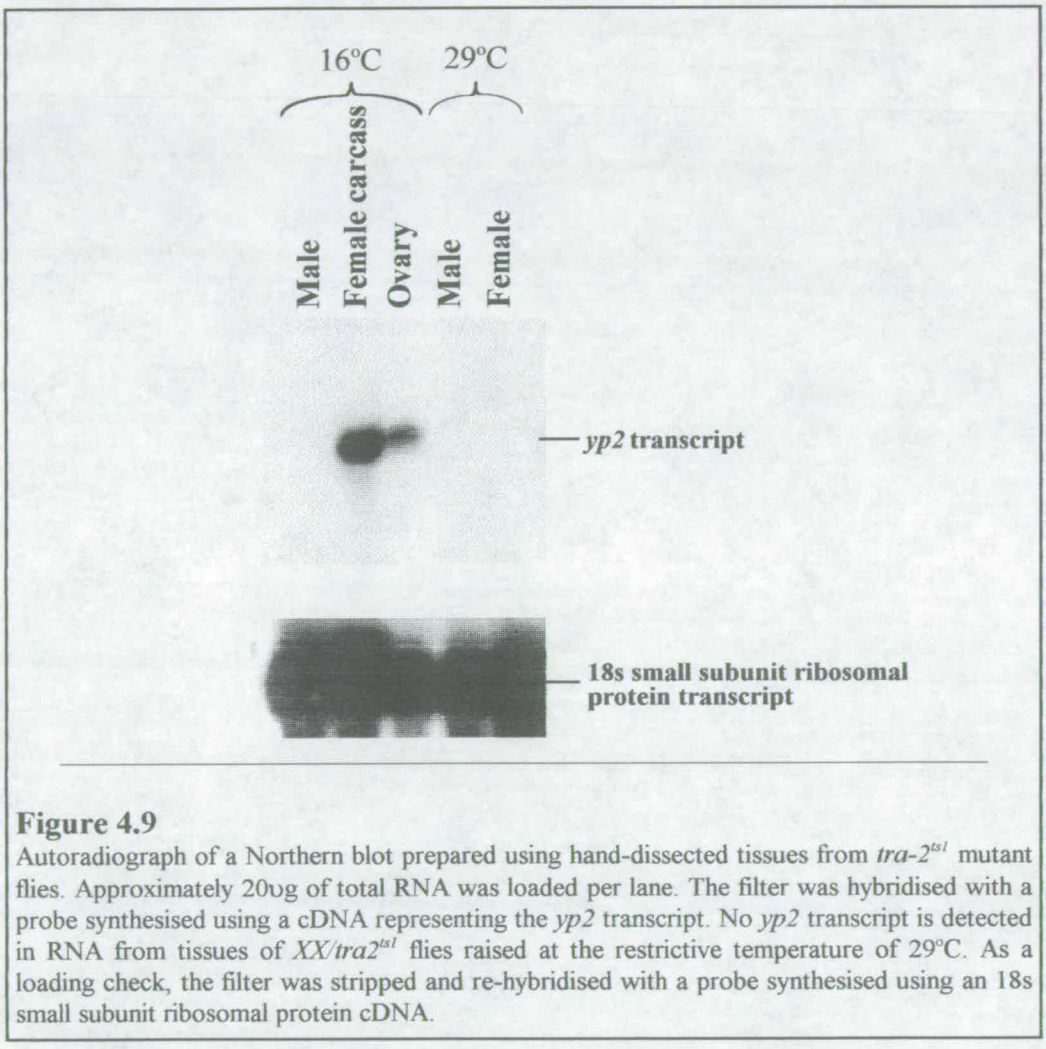


Figure 4.9
Autoradiograph of a Northern blot prepared using hand-dissected tissues from *tra-2*^{ts1} mutant flies. Approximately 20ug of total RNA was loaded per lane. The filter was hybridised with a probe synthesised using a cDNA representing the *yp2* transcript. No *yp2* transcript is detected in RNA from tissues of *XX/tra2*^{ts1} flies raised at the restrictive temperature of 29°C. As a loading check, the filter was stripped and re-hybridised with a probe synthesised using an 18s small subunit ribosomal protein cDNA.

The fact that no *yp* transcripts are detected in XX/*tra-2*^{ts} flies raised at 29°C does not mean that there is no active *tra-2* transcript present in these flies, however. When *XX/tra-2*^{ts} flies are reared at 18°C and then shifted to 29°C for several days, no YP

protein is detected (Bownes *et al.*, 1987). However, female-specific *dsx* transcript can still be detected in these flies (Nagoshi *et al.*, 1988). Thus, there is clearly enough active *tra-2* transcript present in these flies to enable a small amount of splicing to occur at the female-specific acceptor site of *dsx*. However, sufficient levels of *dsx* transcript are not produced to enable de-repression of the *yp* genes. Thus, we cannot rule out a role for *tra-2* in the splicing of *stk61*, since there may be sufficient levels of active *tra-2* transcript present to account for the levels of female carcass-specific transcript present in *XX/tra-2ts flies* raised at 29°C. We can, however, say that the production of the female carcass-specific transcript is not likely to be under direct *dsx* control, as the regulation of this transcript is clearly different from that of the *yp*'s.

4.2.6.3 *tra* regulation of the female carcass-specific transcript.

A Northern blot was prepared using RNA from tra^{FEM} mutant flies (figure 4.4). Flies of the genotype XY/tra^{FEM} ectopically express the female form of *tra* and so develop as pseudofemales. If the production of the *stk61* female carcass-specific transcript is dependent upon Tra/Tra-2, we would expect to see this transcript in the carcass tissue of XY/tra^{FEM} flies. Figure 4.10 shows that XY/tra^{FEM} flies are clearly genetically female, expressing high levels of *yp* transcripts. In fact, higher levels of *yp* expression are seen in XY/tra^{FEM} pseudofemales than in wild type females.

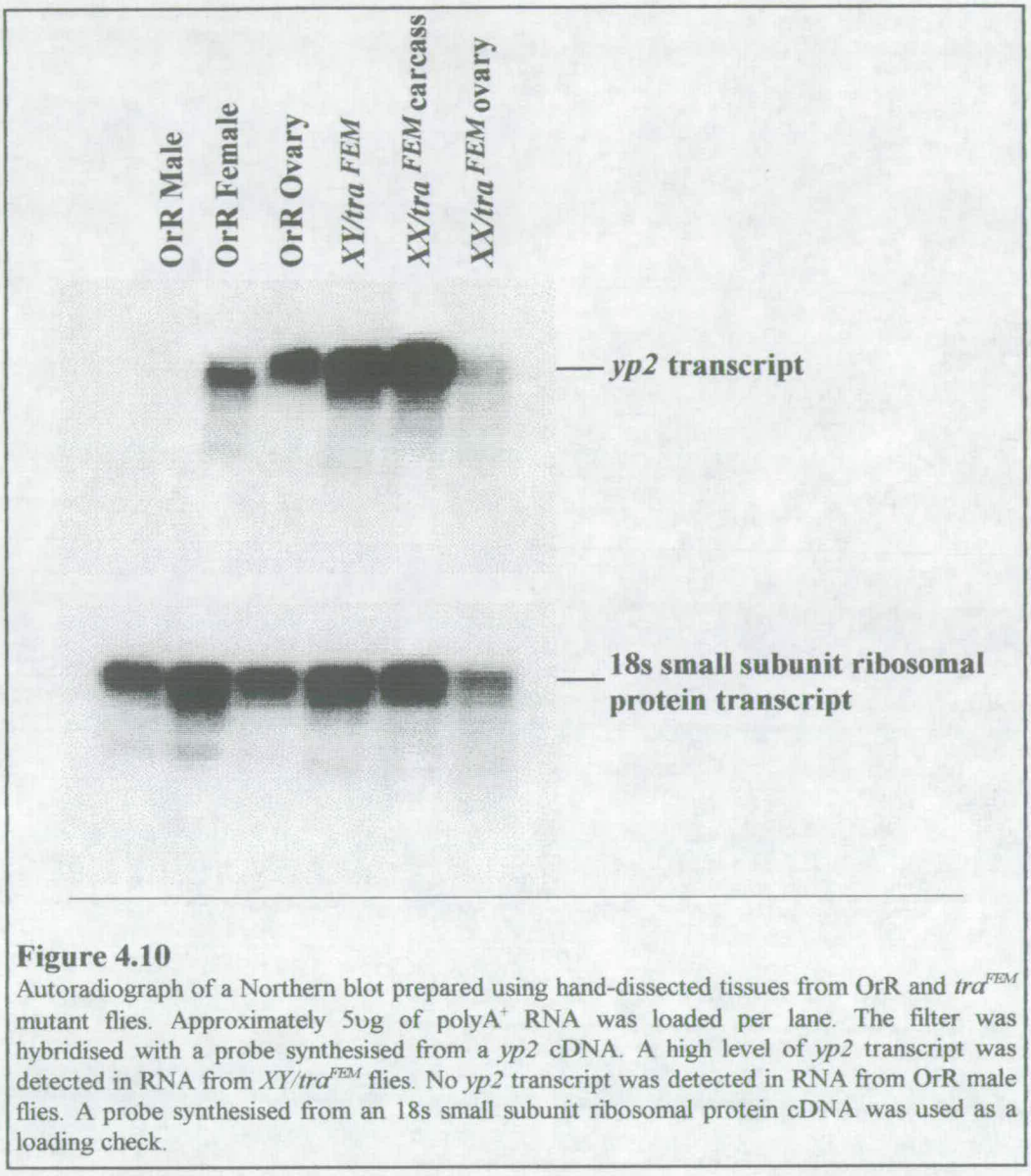


Figure 4.10
Autoradiograph of a Northern blot prepared using hand-dissected tissues from OrR and tra^{FEM} mutant flies. Approximately 5υg of polyA$^+$ RNA was loaded per lane. The filter was hybridised with a probe synthesised from a *yp2* cDNA. A high level of *yp2* transcript was detected in RNA from XY/tra^{FEM} flies. No *yp2* transcript was detected in RNA from OrR male flies. A probe synthesised from an 18s small subunit ribosomal protein cDNA was used as a loading check.

This is in agreement with studies of YP protein expression levels using extracts from tra^{FEM} mutants (M. Bownes, pers. comm.). Again, interpretation of the result shown in figure 4.4 is hampered by the fact that the *stk61* common and female carcass-specific transcripts are poorly separated. The experiment was repeated several times, but good separation between these two transcripts was never observed. However, there is a very noticeable increase in band intensity in the female carcass as opposed to the male lanes (figure 4.4, lanes 1 & 2). The loading check shows that all lanes are equally loaded, and so this increase in band intensity is presumably due

to the presence of the female carcass-specific transcript. Comparing wild type male tissue with carcass tissue from *XY/tra*^{FEM} flies (figure 4.4, lanes 1 & 4), we see the same increase in intensity. In fact, there is an even greater increase in intensity in *XY/tra*^{FEM} flies than with wild type females. These bands have been quantitated, which allows us to visualise these increases in band intensity more clearly (figure 4.11). The loading check bands were also quantified, and these results were used to correct the figures depicted in figure 4.11 for small variations in loading.

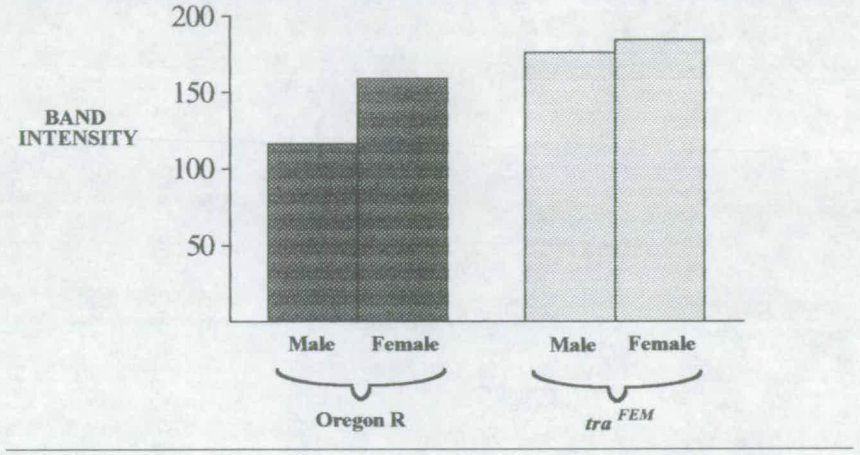


Figure 4.11
Histogram showing intensity of 4.5kb female carcass-specific/common transcript Northern blot bands, as seen in figure 4.4. A significant increase in band intensity is seen in RNA from OrR female carcass tissue relative to OrR male tissue. A similar increase in intensity is seen between RNA from *XY* OrR tissue and *XY/tra*^{FEM} tissue. See text for details.

Application of the χ^2 test shows a probability of 50-70% that these increases are significant, where a probability of >5% is generally taken as indicative of significance. Although the nature of these results means they cannot be taken as conclusive proof, they are strongly indicative of a role for *tra* and *tra-2* in the production of the *stk61* female carcass-specific transcript.

4.3 DISCUSSION

Taken together, the above evidence enables us to propose models for the regulation of the 3.0kb male-specific and 4.5kb female carcass-specific transcripts produced from *stk61*.

Northern analysis suggests a role for the sex determination genes *tra* and *tra-2* in the production of the *stk61* female carcass-specific transcript. cDNA sequence data indicates that this control is direct, with Tra and Tra-2 proteins acting at two *dsx*-like 13-nt repeats to enable the removal of intron 4. RT-PCR analysis was performed using primers which flank intron 4, in an attempt to confirm this hypothesis (data not shown). However, the results from this were inconclusive and a more reliable technique is required to answer this question. One such technique may be RNAase protection, which has not yet been attempted. A riboprobe could be made to a cDNA region including the intron 4 intron/exon boundary. When hybridised to extracted RNA and digested with RNAase, single stranded RNA would be destroyed and species diagnostic of the fully spliced transcript would be produced. If this *tra/tra-2* regulation hypothesis is correct, these diagnostic bands would be seen using RNA from female carcass but not from male carcass.

An RNAase protection-based assay for the presence of the *stk61* female carcass-specific transcript would also enable the *tra/tra-2* regulation of the gene to be demonstrated more conclusively. Northern analysis is not a convenient method to determine the presence of this transcript, due to very low levels of expression and the complication of the common transcript. The rarity of the *stk61* transcripts can be clearly seen by comparison of figures 4.4(a) and 4.9. To produce the bands seen in figure 4.4(a), the filter was hybridised with a gene-specific probe and exposed to film for two weeks. Figure 4.9 shows the same filter, which was stripped and hybridised with a *yp* probe. An exposure of only 2-3 hours was required to produce the *yp* bands shown here. This demonstrates the extreme rarity of the transcript. Other probes, such as *dsx*, were also used (figure 4.8) and no significant problems were

encountered in detecting transcripts from this gene. Thus, Northern blots had to be produced which were sufficiently free of background signal to enable long periods of exposure, as well as being of high sensitivity. Numerous blots were carried out, only a fraction of which yielded useful data. This was further complicated by the inviability of several of the mutants fly stocks. For the *dsx* and *tra-2*^{ts} mutants, trans-heterozygotes had to be constructed, using two separate alleles, to enable sufficient numbers of mutant flies to be obtained. This means that continual repetition of Northern blots becomes a very time consuming process. Thus, it is clear that a more amenable assay for the presence of the female carcass-specific transcript must be developed. RNAase protection should provide a sensitive and reliable assay which would make the characterisation of the *stk61* female carcass-specific transcript less problematic.

The *stk61* male-specific transcript has been shown to be entirely testis-specific. The presence of TCE elements in the 5' UTR make it highly likely that this transcript is germline-specific and is translated late in spermiogenesis. The onset of translation may be coupled to a secondary polyadenylation event which effectively removes the longer 3' UTR seen in cDNA11.

A model whereby Tra and Tra-2 are required for the splicing of *stk61* intron 4 presents difficulties with regard to the testis transcript. The cDNA thought to represent the testis transcript, cDNAa, does not contain intron 4. Tra-2 alone has been shown to be able to promote splicing from the *dsx* female-specific acceptor site (Hoshijima *et al.*, 1991; Ryner & Baker, 1991). Thus, production of the testis transcript in wild type testes, which express *tra-2*, is not unexpected. However, this transcript is also produced at similar levels in the testes of both *XX* and *XY/tra-2*^{ts} mutant flies raised at the restrictive temperature. In this regard, it is interesting to note the presence of a third TCE-like element present in cDNAs a and 11. This element lies at position +2698 in cDNA11, between introns 3 and 4 as shown in figure 4.7, and shows very high homology to the consensus, matching at 11/12 positions. All of the nucleotides which have been identified as important to TCE

function (nt's 5,7,9,10 and 12) match the consensus in this element. We have already considered the possibility that competition between the optimal acceptor site of intron 3 and the sub-optimal site of intron 4 is one reason why utilisation of the intron 4 acceptor site might be an inefficient process. The gel shift and UV-crosslinking experiments previously discussed show that certain testis-specific factors associate with the TCE sequence. Since the third TCE element lies 250bp downstream of the intron 3 acceptor site, it is possible that, in the testis, association of TCE-specific proteins with the element, interferes with the efficiency of the intron 3 acceptor site. This might reduce competition between the sites of introns 3 and 4 to a level where the intron 4 acceptor site is utilised in the absence of *tra* or *tra-2* function. Alternatively, the splicing of the testis-specific *stk61* transcript may be under the control of testis-specific factors. This type of tissue-specific RNA processing is seen in the transposase-encoding transcript from the *Drosophila* P-element, where the intron between open reading frames 2 and 3 is only removed from the primary transcript in the germline (Laski *et al.*, 1986). Thus, active transposase is only produced in this tissue.

In summary, both the testis-specific and female carcass-specific *stk61* transcripts appear to be regulated in a tissue-specific and sex-specific manner. It is likely that both transcripts give rise to active protein in their respective tissues which play differential roles in spermiogenesis and female somatic sex differentiation. Precisely what functions these proteins may be performing will be considered in the next chapter.

CHAPTER 5

SEQUENCE AND BACTERIAL EXPRESSION ANALYSIS OF THE PREDICTED PROTEIN FROM A *DROSOPHILA MELANOGASTER* GENE ENCODING SEX-SPECIFIC TRANSCRIPTS

5.1 INTRODUCTION

In the previous two chapters we have described the cloning of a *Drosophila melanogaster* gene which encodes three different sex-specific transcripts; a 3.0kb testis-specific transcript, a 3.5kb ovary-specific transcript and a 4.5 female carcass-specific transcript. We have discussed evidence which suggests that the testis transcript encodes a protein which is translated late in spermiogenesis and is likely to be involved in the latter stages of sperm differentiation. We have also provided evidence which supports a model whereby the production of the fully-processed female carcass-specific transcript is under direct control of the sex determination genes *tra* and *tra-2*. As yet, we have no direct evidence as to the regulation mechanism of the ovary transcript. These findings suggest that the proteins encoded by the different sex-specific transcripts are functionally active. In this chapter, the possible roles of these proteins in sex differentiation are discussed, including analysis of the predicted protein sequence and bacterial expression studies.

5.2 RESULTS

5.2.1 PREDICTED PROTEIN SEQUENCE ANALYSIS.

All computer sequence analysis was carried out using the University of Wisconsin Genetics Computer Group, Sequence Analysis Software Package, Version 7 (Devereux *et al.*, 1984). The predicted protein primary sequence encoded by cDNAs a and 11 is shown in figure 5.1. In an attempt to assign a possible function to this protein, this sequence was used to perform exhaustive computer searches of the Genbank gene database. Initially, no significant homologies to known gene products were identified. Since it is possible that small areas of homology could be missed when using the entire sequence as a basis for comparison, the computer searches were repeated using smaller sections of the sequence. This method revealed that certain short runs of amino acids within the sequence were highly homologous to parts of protein kinase catalytic domains from a number of different genes. Using a mixture of computer searching and alignment by eye, it was found that the predicted protein from cDNAs a and 11 contained a complete kinase catalytic domain.

The eleven subdomains (I-XI) which make up this catalytic domain, are indicated in figure 5.1. Thus, the transcripts produced from *stk61* encode a novel protein kinase. In a comparative study of 38 serine/threonine-specific and 27 tyrosine-specific protein kinases, it was found that these eleven regions were present in all 65 primary protein sequences (Hanks *et al.*, 1988). One of the most interesting regions of homology lies in subdomain VI, where the consensus sequence D-L-A-A-R-N is diagnostic of tyrosine-specific protein kinases, while the consensus D-L-K-P-E-N indicates serine/threonine-specificity. In figure 5.1, we can see that subdomain VI contains the sequence D-L-K-P-E-N indicating that this protein is a serine/threonine-specific protein kinase (S/T kinase). Within the catalytic domain, 19 residues were found to be invariant or nearly invariant in all of the S/T kinases analysed. All of these amino acids are conserved in the predicted protein from *stk61* and have been boxed in figure 5.1. A further 18 positions within the domain

exhibited amino acids of similar chemical nature in all 38 S/T kinases. Again, the nature of these residues is conserved within the primary sequence of STK61 protein and their positions have been underlined in figure 5.1.

```
1    MAKEKASATV SLGESNFRDI NLKDLAVVVE AASRLHHQQN VCGCGAVSST

51   ENNNNSRYGS SKYLTNGHTS PLAAAVASNS SSVATTPHCR MLHNCSLQQY

101  QNDIRQQTEI LDMLRHEHQQ GYQSQQQQQQ PQQQQEQQQQ QEQSQQQQQL

                    I                                  II
151  QNPAPRRSPN DFIFGRYIGE GSYSIVYLAV DIHSRREYAI KVCEKRLILR

              III                IV                  V
201  ERKQDYIKRE REVMHQMTNV PGFVNLSCTF QDQRSLYFVM TYARKGDMLP

                              VI
251  YINRVGSFDV ACTRHYAAEL LLACEHMHRR NVVHRDLKPE NILLDEDMHT

               VII
301  LIADFGSAKV MTAHERALAT EHCSEQRRSN SDEDDEDSDR LENEDEDFYD

                                                  VIII
351  RDSEELDDRD DEQQQEEMDS PRHRQRRYNR HRKASFVGTA QYVSPEVLQN

                    IX                                 X
401  GPITPAADLW ALGCIVYQMI AGLPPFRGSN DYVIFKEILD CAVDFPQGFD

                              XI
451  KDAEDLVRKL LRVDPRDRLG AQDEFGYYES IRAHPFFAGI DWQTLRQQTP

501  PPIYPYLPGV SQDEDFRSSY TVPGDLEPGL DERQISRLLS AELGVGSSVA

551  MPVKRSTAKN SFDLNDAEKL QRLEQQKTDK WHVFADGEVI LKKGFVNKRK

601  GLFARKRMLL LTTGPRLIYI DPVQMIKKGE IPWSPDLRAE YKNFKIFFVH

651  TPNRTYYLDD PEGYAIHWSE AIENMRKLAY GDPSSTSAVS CSSGSSNSLA

701  VISNSSAASS SNSPTVKRSS PVNAPQASTA SDNRTLGSTR TGTSPSKKTA

751  SK
```

Figure 5.1
Predicted sequence of STK61 protein. Serine/threonine-specific protein kinase catalytic subdomains I-XI are indicated. Residues which were found to be invariantly conserved in a study of 38 S/T kinases (see main text for details) are boxed. Underlining indicates positions where amino acids of similar chemical character are found.

These conserved residues are thought to play a pivotal role in the functioning of the catalytic domain (Hanks *et al.*, 1988). The conserved glycine and valine residues in subdomain I (boxed in figure 5.1) have been proposed to be involved in the binding of ATP in the tyrosine-specific kinase (Y kinase) Src. Mutation of the conserved lysine in subdomain II abolishes the kinase activity of Src and is believed to be involved in the transfer of the phosphor group from ATP. The conserved aspartate (D) and asparagine (N) residues in subdomain VI, and the conserved "DFG" triplet in subdomain VII are also thought to be involved in ATP binding. In Src, the three residues representing the conserved "APE" triplet in subdomain VIII (corresponding to "SPE" in figure 5.1) are all required for kinase function.

It is difficult to propose what function the protein shown in figure 5.1 may be carrying out on the basis of homology to other protein kinases. This is because the homology within the kinase domain is highly localised and no significant homology is seen outwith the catalytic domains. The subdomains of the kinase catalytic domain which show the greatest levels of conservation are subdomains I, II, VI, VII, and VIII. Since some of the conserved residues within these subdomains have been shown to be required for kinase activity, it is likely that these domains are responsible for the regulation and catalytic activity of the active site, while the other, less conserved, regions from less critical structural components of the protein. Thus, in an attempt to categorise the predicted STK61 protein, the sequences of subdomains I, II, VI, VII and VIII were compared with those from a variety of different kinases know to undergo distinct forms of regulation and perform disparate functions (figure 5.2).

Figure 5.2(a)

Predicted protein sequence comparison of STK61 catalytic kinase domains with those from 21 other S/T kinases. Positions of identity to STK61 are highlighted. For each sequence, the number and percentage of identities to STK61 are shown. cAPK-α/β=bovine cAMP-dependent kinase subunits, SRA3=Yeast suppressor of Ras cAMP-dependent kinase, PKC-α/β/γ=Bovine protein kinase C subunits, CaMII-α/β=Rat Ca^{2+}/calmodulin-dependent kinase subunits, PhK-γ=Rabbit phosphorylase kinase subunit, SNF1=Yeast "sucrose nonfermenting" kinase, Nim1=Yeast "new inducer of mitosis" kinase, KIN1=Putative yeast protein kinase, CDC28=Yeast cell-division-cycle 28 kinase, CDC2=Yeast cell-division-cycle 2 kinase, CDC2Hs=Human homolog of yeast CDC2, Raf=Human Raf kinase, A-Raf=Human Raf-related oncogenic kinase, PKS=Human Raf-related cellular kinase, DCKII=*Drosophila* casein kinase II α subunit, CDC7=Yeast cell-division-cycle 7 kinase, STE7=Yeast "Sterile" 7 kinase.

Subdomain I		Identities	
cAPK-α	DQFERIKTLGTGSFGRVMLVKHME	6	25%
cAPK-β	GDFERKKTLGTGSFGRVMLVKHKA	7	29%
SRA3	KNFQILRTLGTGSFGRVHLIRSRH	8	33%
PKC-α	TDFNFLMVLGKGSFGKVMLADRKG	9	37%
PKC-β	TDFNFLMVLGKGSFGKVMLSERKG	8	33%
PKC-γ	SDFSFLMVLGKGSFGKVMLAERRG	9	37%
CaMII-α	EEYQLFEELGKGAFSVVRRCVKVL	5	21%
CaMII-β	DEYQLYEDIGKGAFSVVRRCVKLC	6	25%
PhK-γ	ENYEPKEILGRGVSSVVRRCIHKP	4	17%
SNF1	GNYQIVKTLGEGSFGKVKLAYHTT	7	29%
Nim1	GVWRLGKTLGTGSTSCVRLAKHAK	8	33%
KIN1	GDWEFVETVGAGSMGKVKLAKHRY	8	33%
CDC28	ANYKRLEKVGEGTYGVVYKALDLR	8	33%
CDC2	ENYQKVEKIGEGTYGVVYKARHKL	8	33%
CDC2Hs	EDYTKIEKIGEGTYGVVYKGRHKT	8	33%
Raf	SEVMLSTRIGSGSFGTVYKGKWHG	6	25%
A-Raf	SEVQLLKRIGTGSFGTVFRGRWHG	5	21%
PKS	SEVQLLKRIGTGSFGTVFRGRWHG	5	21%
DCKII	DDYQLVRKLGRGKYSEVFEAINIT	9	37%
CDC7	NEYKLIDKIGEGTFSSVYKAKDIT	11	46%
STE7	QDLVQLGKIGAGNSGTVVKALHVP	6	25%
STK61	NDFIFGRYIGEGSYSIVYLAVDIH	24	100%

(continued...)

Figure 5.2(b)

Subdomain II		Identities	
cAPK-α	TGNHYAMKILDKQKVV	4	25%
cAPK-β	TEQYYAMKILDKQKVV	4	25%
SRA3	NGRYYAMKVLKKEIVV	6	38%
PKC-α	TEELYAIKILKKDVVI	5	31%
PKC-β	TDELYAVKILKKDVVI	4	25%
PKC-γ	SDELYAIKILKKDVIV	7	44%
CaMII-α	AGQEYAAKIINTKKLS	4	25%
CaMII-β	TGHEYAAKIINTKKLS	4	25%
PhK-γ	TCKEYAVKIIDVTGGG	4	25%
SNF1	TGQKVALKIINKKVLA	3	19%
Nim1	TGDLAAIKIIPIR---	4	25%
KIN1	TNEVCAVKIVNRATKA	2	13%
CDC28	GQRVVALKKIRLESED	3	19%
CDC2	SGRIVAMKKIRLEDES	4	25%
CDC2Hs	TGQVVAMKKIRLESEE	2	13%
Raf	---DVAVKILKVVDPT	2	13%
A-Raf	---DVAVKVLKVSQPT	3	19%
PKS	---DVAVKVLKVSQPT	3	19%
DCKII	TTEKCVVKILKPVKKK	1	6%
CDC7	GSNYVALKKIYVTS--	2	13%
STE7	DSKIVAKKTIPVEQNN	2	13%
STK61	SRREYAIKVCEKRLIL	16	100%

(continued...)

Figure 5.2(c)

Subdomain VI			Identities	
cAPK-α	FSEPHARFYAAQIVLTFEVLHSL-DLITRDLKPENLLIDQQG	17	40%
cAPK-β	FSEPHARFYAAQIVLTFEYLHSL-DLIYRDLKPENLLIDHQG	17	40%
SRA3	FPNPVAQIFAAEVCLALEYLHSK-DIIYRDLKPENILLDKNG	19	45%
PKC-α	FKEPQAVFYAAEISIGLFFLHKR-GIIYRDLKLDNVMLDSEG	14	33%
PKC-β	FKEPHAVFYAAEIAIGLFFLQSK-GIIYRDLKLDNVMLDSEG	12	29%
PKC-γ	FKEPHAAFYAAEIAIGLFFLHNQ-GIIYRDLKLDNVMLDAEG	13	31%
CaMII-α	YSEADASHCIQQILEAVLHCHQM-GVVHRDLKPENLLLASKL	18	43%
CaMII-β	YSEADASHCIQQILEAVLHCHQM-GVVHRDLKPENLLLASKL	18	43%
PhK-γ	LSEKETRKIMRALLEVICALHKL-NIVHRDLKPENILLDDDM	21	50%
SNF1	MSEQEARRFFQQIISAVEYCHRH-KIVHRDLKPENLLLDEHL	18	43%
Nim1	LSEREAAHYLSQILDAVAHCHRF-RFRHRDLKLENILIKVNE	16	38%
KIN1	IREHQARKFARGIASALIYLHAN-NIVHRDLKIENIMISDSS	14	33%
CDC28	LGADIVKKFMMQLCKGIAYCHSH-RILHRDLKPQNLLINKDG	11	26%
CDC2	LDPRLVQKFTYQLVNGVNFCHSR-RIIHRDLKPQNLLIDKEG	13	31%
CDC2Hs	MDSSLVKSYLYQILQGIVFCHSR-RVLHRDLKPQNLLIDDKG	15	36%
Raf	FQMFQLIDIARQTAQGMDTLHAK-NIIHRDMKSNNIFLHEGL	12	29%
A-Raf	FDMVQLIDVARQTAQGMDYLHAK-NIIHRDLKSNNIFLHEGL	14	33%
PKS	FDMVQLIDVARQTAQGMDYLHAK-NIIHRDLKSNNIFLHEGL	14	33%
DCKII	LTDYEIRYYLFELLKALDYCHSM-GIMMRDVKPHNVMIDHEN	13	31%
CDC7	LPIKGIKKYIWELLRALKFVHSK-GIIHRDIKPTNFLFNLEL	13	31%
STE7	FNELTISKIAYGVLNGLDNLYRQYKIIHRDIKPSNVLINSKG	11	26%
STK61	FDVACTRHYAAELLLACEHMHRR-NVVHRDLKPENILLDEDM	42	100%

(continued...)

Figure 5.2(d)

Subdomain VII		Identities	
cAPK-α	YIQYTDFGFAKRVKGRT--	5	26%
cAPK-β	YIQYTDFGFAKRVKGRT--	5	26%
SRA3	HIKITDFGFAKYVPDVI--	7	37%
PKC-α	HIKIADFGMCKEHMMDGVT	7	37%
PKC-β	HIKIADFGMCKENIWDGVT	7	37%
PKC-γ	HIKITDFGMCKENVFPGST	6	32%
CaMII-α	AVKLADFGLAIEVEGEQQA	6	32%
CaMII-β	AVKLADFGLAIEVQGDQQA	6	32%
PhK-γ	NIKLTDFGFSCQLDPGEKL	4	21%
SNF1	NVKIADFGLSNIMTDGNFL	7	37%
Nim1	QIKIADFGMATVEPNDSCL	7	37%
KIN1	EIKIIDFGLSNIYDSRKQL	4	21%
CDC28	NLKLGDFGLARAFGVPLRA	6	32%
CDC2	NLKLADFGLARSFGVPLRN	6	32%
CDC2Hs	TIKLADFGLARAFGIPIRV	6	32%
Raf	TVKIGDFGLATVKSRWSGS	6	32%
A-Raf	TVKIGDFGLATVKTRWSGA	8	42%
PKS	TVKIGDFGLATVKTRWSGA	8	42%
DCKII	KLRLIDWGLAEFYHPGQEY	3	16%
CDC7	RGVLVDFGLAEAQMDYKSM	4	21%
STE7	QIKLCDFGVSKKLINSIA-	4	21%
STK61	HTLIADFGSAKVMTAHERA	19	100%

(continued...)

Figure 5.2(e)

Subdomain VIII		Identities		Overall
cAPK-α	WTLCGTPEYLAPEII	5	33%	32%
cAPK-β	WTLCGTPEYLAPEII	5	33%	33%
SRA3	YTLCGTPDYIAPEVV	6	40%	40%
PKC-α	RTFCGTPDYIAPEII	6	40%	35%
PKC-β	KTTCGTPDYIAPEII	5	33%	31%
PKC-γ	RTFCGTPDYIAPEII	6	40%	35%
CaMII-α	FGFAGTPGYLSPEVL	9	60%	35%
CaMII-β	FGFAGTPGYLSPEVL	9	60%	36%
PhK-γ	REVCGTPSYLAPEII	5	33%	32%
SNF1	KTSCGSPNYAAPEVI	5	33%	34%
Nim1	ENYCGSLHYLAPEIV	4	27%	33%
KIN1	HTFCGSLYFAAPELL	5	33%	28%
CDC28	THEIVTLWYRAPEVL	6	40%	28%
CDC2	THEIVTLWYRAPEVL	6	40%	31%
CDC2Hs	THEVVTLWYRSPEVL	8	53%	33%
Raf	EQPTGSVLWMAPEVI	4	27%	24%
A-Raf	EQPSGSVLWMAAEVI	3	20%	28%
PKS	EQPSGPVLWMAAEVI	3	20%	28%
DCKII	NVRVASRYFKGPELL	4	27%	23%
CDC7	ANRAGTRGFRAPEVL	7	47%	29%
STE7	DTFVGTSTYMSPERI	8	53%	27%
STK61	ASFVGTAQYVSPEVL	15	100%	100%

The invariant or nearly invariant residues indicated in figure 5.1 are highly conserved throughout all of the kinases shown in figure 5.2. These residues are likely to be essential for the catalytic domain to perform it's kinase function. It is clear from the comparison in figure 5.2 that residues other than these invariant ones are much less well conserved between members of different families of kinases. Thus, it is reasonable to assume that significant homology in residues other than the invariant ones is an indicator of kinase function. The levels of homology which the kinases shown in figure 5.2 exhibit to STK61 varies from subdomain to subdomain. For example, in subdomain I, CDC7 has 46% identity to STK61. This is a very high level, relative to the other proteins examined. However, CDC7 shows average to low levels of homology throughout the rest of the subdomains. It may be that subdomain I is required for some shared regulatory mechanism in both CDC7 and STK61. Generally, the highest levels of homology to STK61 are seen by members of the cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinase families. Subdomain VI exhibits the highest number of conserved residues of all the subdomains (figure 5.2(c)). In this subdomain, the Ca^{2+}/calmodulin-dependent phosphoylase kinase γ subunit (PhK-γ) shows 50% identity with STK61, and the cAMP-dependent Ras suppressor kinase (SRA3) shows 45% identity. The lowest level of homology shown with this subdomain is to the STE7 kinase, at 24%.

Between the N-terminal end of STK61 and the start of the kinase catalytic domain there are several runs of the acidic amino acid glutamine (Q). These Q-repeats have been termed OPA repeats and are found in a number of developmentally important proteins, including kinases. However, no OPA repeat-containing kinases have been identified from *Drosophila* or indeed from any dipteran insect. Initially discovered in the *Notch* gene (Wharton *et al.*, 1985; Grabowski *et al.*, 1991), OPA-encoding repeats have been seen in a number of different genes which have functions in development, including *twist*, *hunchback* and *dorsal*. Although little is known about what function OPA repeats may have, the proteins in which they are found are developmentally and/or spatially regulated, being involved in tissue-specific functions. Thus, we might expect *stk61* to be expressed in a tissue-specific manner.

However, *in situ* RNA hybridisations performed using probes synthesised from cDNAa to whole mount carcass tissue and imaginal discs show expression in a global non tissue-specific manner (data not shown). This is probably due to the presence of the non tissue/sex-specific common transcript and it will only be when antibody *in situs* are done that the true expression pattern of *stk61* is revealed.

Since no significant homology is seen to between STK61 and other known protein kinases, other than short stretches of globally-conserved residues within the catalytic domain, together with the fact that no other *Drosophila* OPA repeat-containing kinases are known, it is reasonable to suppose that the *stk61* gene encodes a member of a novel family of OPA repeat-containing S/T kinases.

The predicted STK61 protein is of a fairly neutral pH, having a pKa of 6.59 (figure 5.3). The secondary structure of STK61 was predicted using Chou/Fasman and Kyle/Doolittle algorithms as shown in figures 5.4 and 5.5. This analysis indicates that the STK61 protein is cytosolic since no hydrophobic transmembrane repeats are seen. This is unsurprising, since the vast majority of S/T kinases are cytosolic. In figure 5.5, the hydrophilic OPA repeats are clearly seen covering amino acids 119-151. The catalytic domain begins 8 amino acids down from this hydrophilic stretch in the C-terminal direction and extends over amino acids 160-490. The catalytic domain is preceded by a short break of α-helix, after which subdomains I-VII consist predominantly of β-sheet. There is a large spacer region between subdomains VII and VIII covering amino-acids 318-383. In figure 5.5 it is clear that this spacer region is extremely hydrophilic and contains a large quantity of α-helix. Subdomain VIII begins after this hydrophilic α-helix spacer and the remainder of the catalytic domain consists of 4 stretches of β-sheet separated by short α-helical regions. After the end of the catalytic domain, the protein C-terminus is largely α-helix and exhibits no striking areas of high hydrophobicity or hydrophilicity. Similarly, the N-terminus of the protein, prior to the OPA repeats, is mainly α-helical. It appears, therefore, that the STK61 protein is divided up into areas by it's secondary structure which seem to demarcate the catalytic areas of the protein.

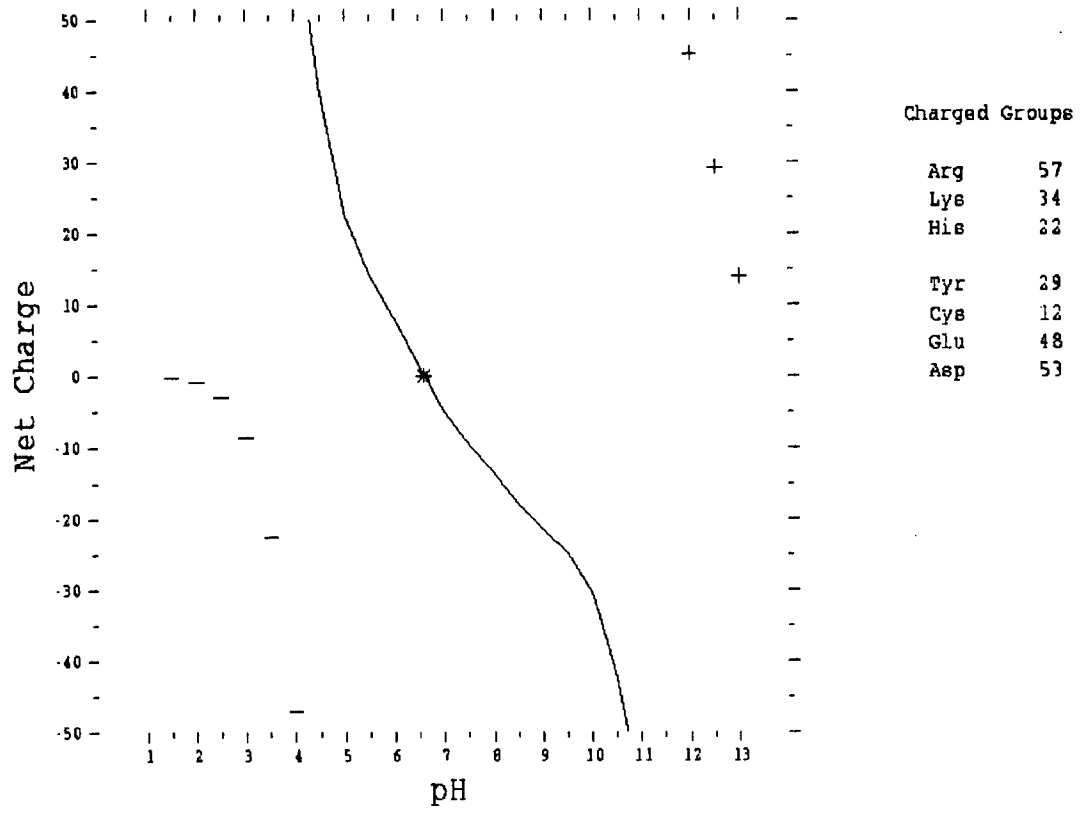


Figure 5.3
Isoelectric plot of the STK61 predicted protein sequence.

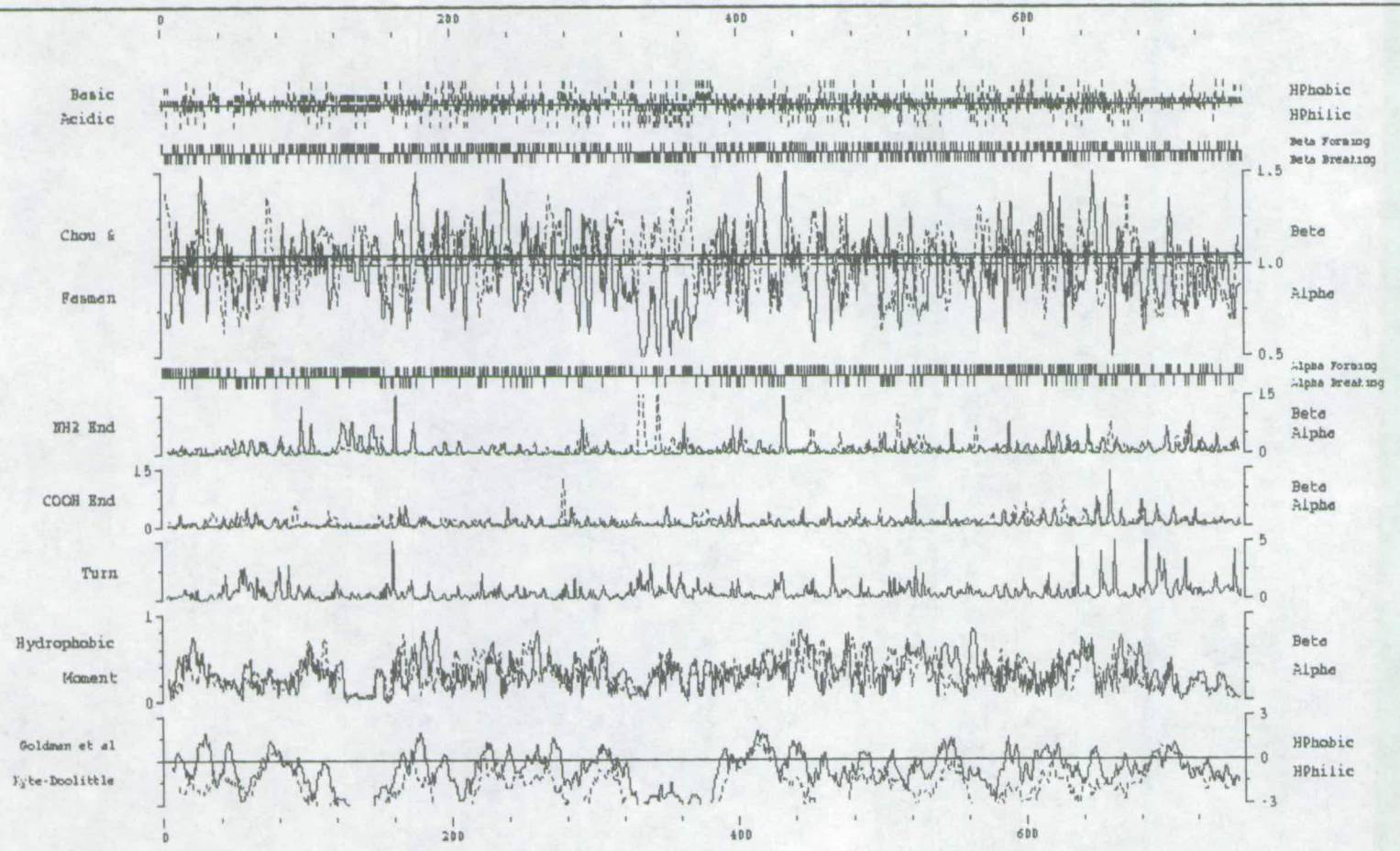


Figure 5.4

Computer analysis of STK61 predicted protein sequence. Plots indicate hydrophobic and hydrophilic regions, as well as areas of α-helix and β-sheet. A pictorial representation of this data is shown in figure 5.5.

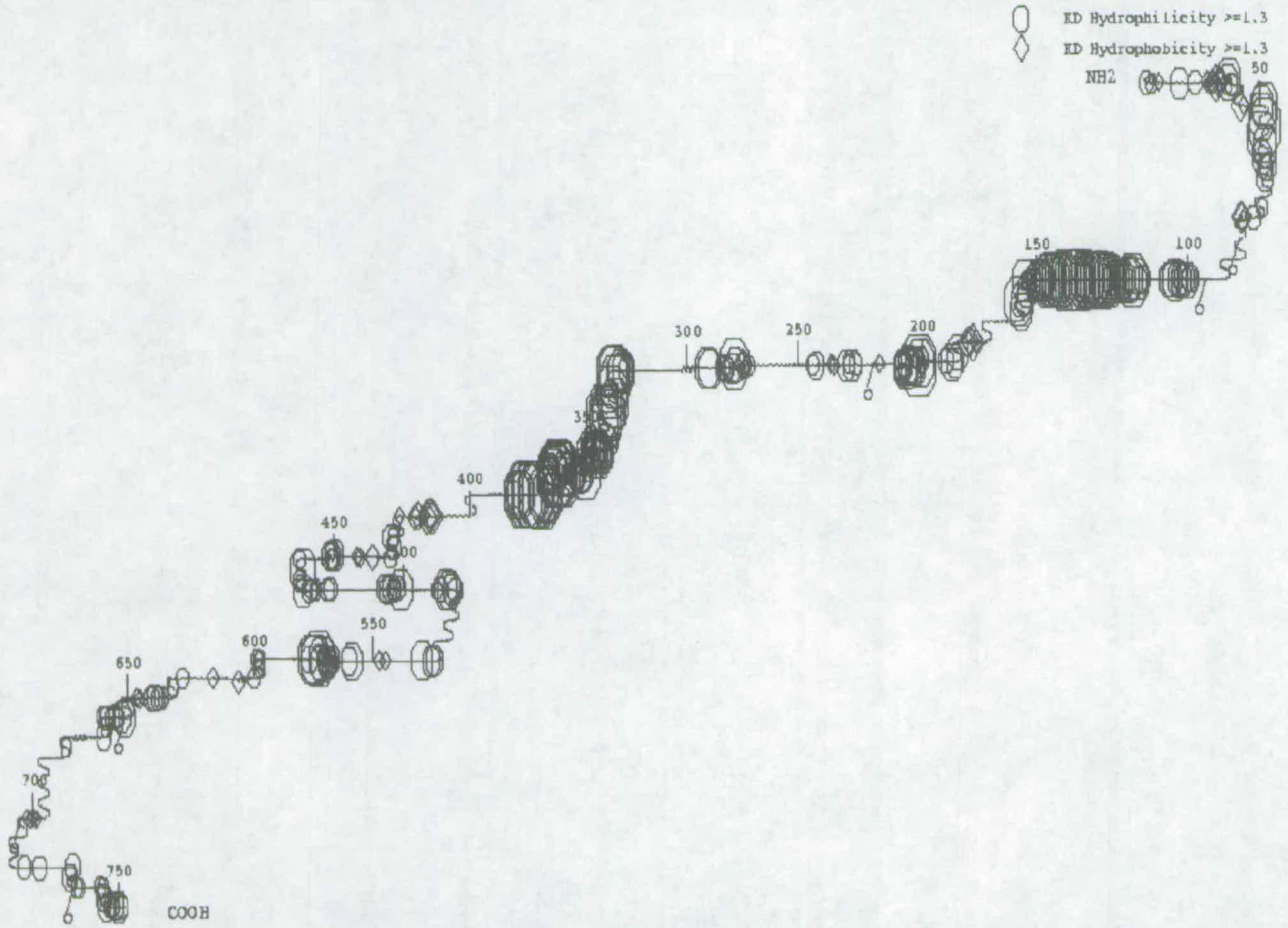


Figure 5.5

Pictorial representation of data presented in figure 5.4. Hydrophobic and hydrophilic regions are indicated. Areas which are predicted to consist of α-helix or β -sheet are also shown.

The region containing the kinase catalytic domain and OPA repeats is bounded by α-helical C and N-termini. The catalytic domain itself consists mainly of β-sheet, with a large hydrophilic α-helix spacer between subdomains VII and VIII. It seems likely that the catalytic domain, hydrophilic OPA repeats and hydrophilic spacer region present on the surface of the protein and fold together to form the active site, with the less hydrophilic α-helical termini forming the internal structure of the protein.

5.2.2 BACTERIAL EXPRESSION STUDIES.

5.2.2.1 Codonpreference analysis of the *stk61* open reading frame.

The above analysis is based on the predicted protein sequence encoded by cDNAs a and 11. However, it is not known whether this protein is actually produced *in vivo*. Figure 5.6 (overleaf) shows a codon preference analysis of cDNA11. This graph indicates the probability that the codons used in the cDNA11 (and therefore cDNAa) ORF are typical of codons generally used in *Drosophila* genes. It is clear from this that the ORF of cDNAa and cDNA11 contains typical *Drosophila* codons and so is highly likely to be translated *in vivo*.

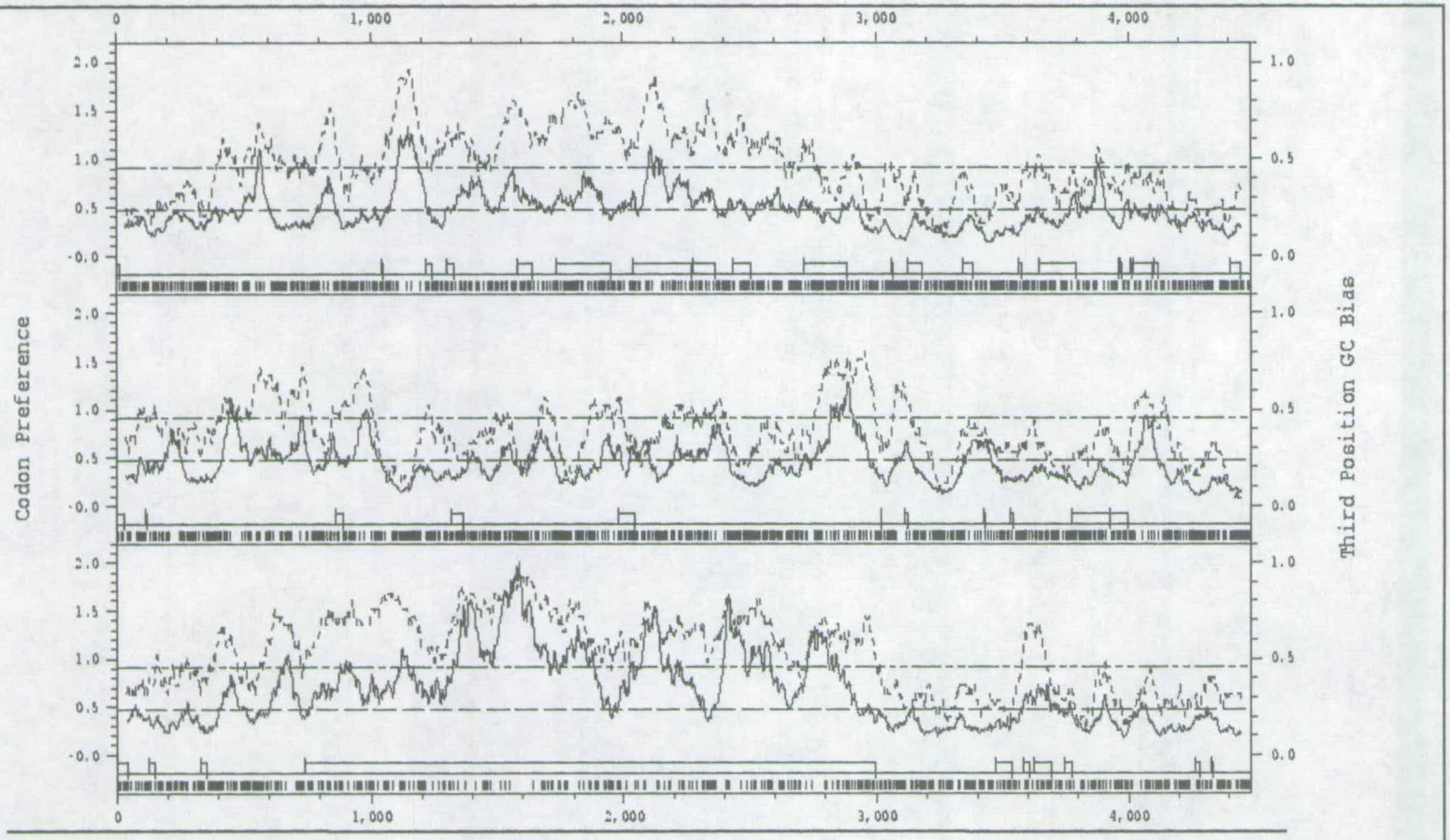


Figure 5.6

Codonpreference computer analysis of cDNA11. The lower panel shows the correct reading frame for translation of the STK61 protein The long STK61-encoding open reading frame is shown as an open box at the bottom of this panel. The lower trace in each panel indicates the frequency with which a particular codon is used in *Drosophila*, with values above the lower line indicating typical codons. The lower panel shows that the cDNA11 open reading frame utilises typical *Drosophila* codons. A similar result is seen for G/C bias (upper line and upper trace in each panel).

5.2.2.2 Construction of pGEX^{STK61}.

To determine whether the cloned cDNAs are in fact capable of producing the predicted protein, the ORF from cDNAa was cloned into a bacterial expression vector. Figure 5.7 shows the cloning strategy that was used and the composition of the final construct, pGEX^{STK61}. The pGEX series of GST-fusion vectors were used for this analysis due to the ease of detection and purification of GST-fusion proteins via anti-GST immunofluorescence and GST-affinity chromatography.

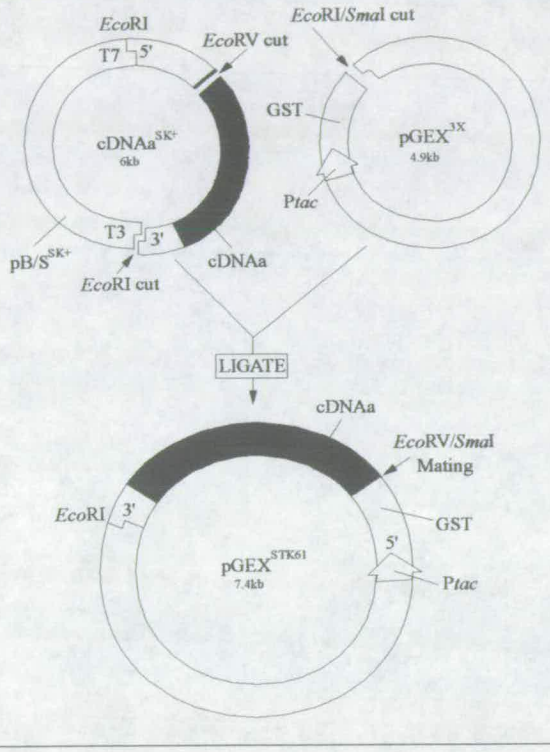


Figure 5.7
Construction of pGEX^{STK61} expression construct. cDNAa open reading frame is shaded. Relevant restriction sites are shown. GST=Glutathione-S-transferase, pB/S^{SK+}=pBluescript SK^{+} plasmid vector, P*tac*=*tac* IPTG-inducible promoter. cDNAa^{SK+} was digested with *Eco*RI and partially digested with *Eco*RV, to produce the fragment shown, which was ligated into pGEX^{3X} to create pGEX^{STK61}. The cDNAa ORF in pGEX^{STK61} lacks the codons encoding the 19 amino acids at the extreme amino-terminus of the predicted STK61 protein. The resulting pGEX^{STK61} construct encodes STK61 protein with GST sequence fused to its amino terminus.

5.2.2.3 Bacterial expression of STK61/GST fusion protein.

The results of initial expression analysis are shown in figure 5.8. The cDNA ORF which was cloned into the expression vector encodes a predicted STK61 protein of 83.4kDa. Therefore, when fused to the 26kDa GST protein, the full length protein encoded by $pGEX^{STK61}$ has a predicted molecular weight of 109.4 kDa.

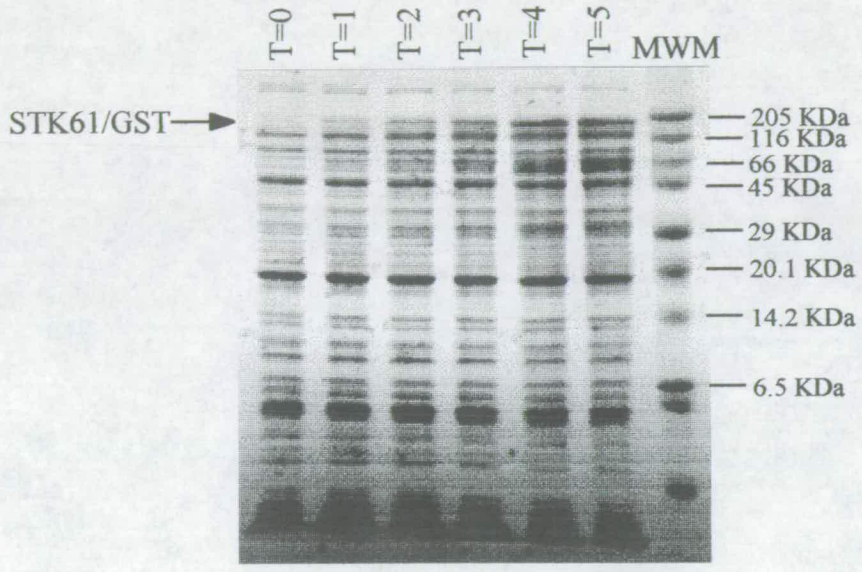


Figure 5.8
SDS PAGE of protein extracts from bacterial cells transformed with $pGEX^{STK61}$ expression construct. MWM=Molecular weight markers. Samples were taken from the expression culture prior to IPTG-induction (T=0) and at hourly intervals after induction (T=1,2,3,4,5). The optical density of each sample was determined and used to correct for the increasing density of the culture through time. Thus, each lane shown above represents protein extracted from an approximately equal number of cells. The band representing induced STK61/GST fusion protein is indicated. This band appears larger than expected (109.4 KDa). This may be due, in part, to differences in salt concentrations between the marker and bacterial extract lanes. However, some post-translational modification (such as glycosylation) may also have occurred, increasing the observed molecular weight of the fusion protein.

One hour after IPTG-induction an induced band of >100kDa is clearly visible, indicating that the full length STK61 protein is being synthesised. Smaller bands are also induced, indicating that some proteolytic degradation of the expressed protein is occurring.

5.2.2.4 Western blot analysis of STK61/GST fusion protein.

Anti-GST antibody was used to perform Western analysis to confirm that the induced band was being produced from the pGEX^{STK61} construct (figure 5.9). The 26kDa GST protein produced from pGEX^{3X} is well expressed and is detected very specifically by anti-GST as shown in figure 5.9.

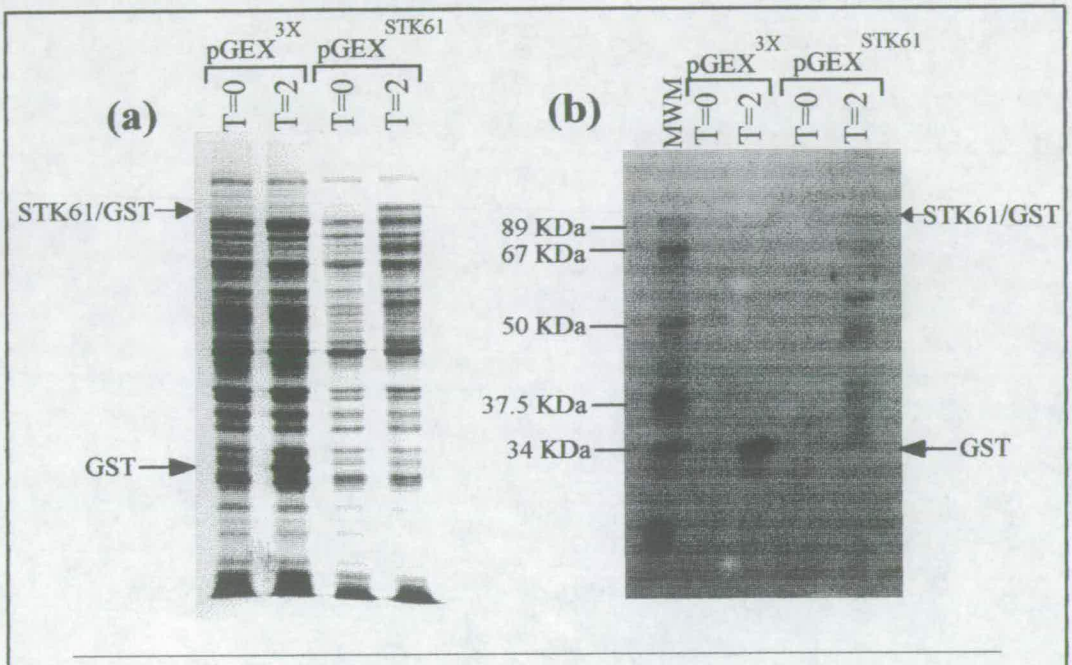


Figure 5.9
(a) SDS PAGE of protein extracts from pGEX^{3X} and pGEX^{STK61} transformed cells before (T=0) and after (T=2) IPTG-induction. Both pGEX^{3X}-derived and pGEX^{STK61}-derived proteins can be seen in the induced extracts. (b) Anti-GST Western blot of protein gel shown in (a). pGEX^{3X}-derived GST protein is only detected in the extract from induced cells. Similarly, no STK61/GST fusion protein is detected in un-induced pGEX^{STK61}-transformed cells. Extracts from pGEX^{STK61}-transformed induced cells clearly contain STK61/GST fusion protein. Only a very small amount of this protein is full-length. Nine GST-containing STK61/GST proteolytic degradation products are detected. MWM=Molecular Weight Markers.

Expression of the fusion protein from pGEX^{STK61} is at quite a low level, however, although it is still detected by anti-GST. Nine smaller bands are also detected by anti-GST, indicating that the STK61/GST fusion is undergoing significant levels of proteolysis. It is most likely that this proteolysis is occurring while the protein is being expressed in culture, as cells were frozen in liquid Nitrogen immediately after harvesting and anti-proteolytic chemicals were used in all subsequent steps

(Materials & Methods). Different host strains were also used but showed similar levels of proteolysis (data not shown). Similarly, attempts to reduce proteolysis by culturing cells at low temperature (25°C-30°C) proved ineffective.

Levels of $pGEX^{STK61}$ expression are much lower than levels of expression of the 26kDa GST protein from $pGEX^{3X}$. This could partly be explained by the much larger size of the $pGEX^{STK61}$-encoded protein but could also be due to deleterious effects of the STK61 kinase function on the bacterial cells. To maximise levels of fusion protein expression, freshly-transformed cells were used for each expression experiment. Cultures were grown from single-cell inoculations to Log-phase when they were induced with IPTG. This strategy was used in preference to inoculation from overnight cultures to prevent the cultures ever reaching turbidity, since the competition this would cause between cells may increase the incidence of $pGEX^{STK61}$ plasmid loss. To prevent uninduced expression from the $pGEX^{STK61}$ construct, BL21 DE3 LysS cells were also used as hosts. This strain contains the LyS plasmid which prevents expression from the vector promoter in the absence of IPTG induction (Materials and Methods). However, even lower levels of $pGEX^{STK61}$ expression were seen with these cells and so XL-1 cells were used in preference.

5.2.2.5 Kinase assays of crude protein extracts containing STK61/GST protein.

In an attempt to ascertain whether the protein expressed from $pGEX^{STK61}$ was functionally active, preliminary kinase assays were carried out. Essentially, this was done by preparing crude bacterial extracts from IPTG-induced or IPTG-uninduced cells containing either $pGEX^{STK61}$, or $pGEX^{3X}$ vector. γ-radiolabelled ATP was then added to the extracts which were incubated for 1 hour at 25°C and then analysed by SDS-PAGE and autoradiography (Materials & Methods). A typical result is shown in figure 5.10. Although it is clear that general kinase activity is occurring in these extracts, no increase in kinase activity is seen in extracts from cells which contain $pGEX^{STK61}$, either induced or uninduced. Figure 5.6 shows that the ORF which was cloned into $pGEX^{3X}$ does not encode the first 19 amino acids of the predicted STK61

protein. However, the predicted secondary structure of the protein, as discussed above, suggests that these residues are unlikely to be critical to the protein's function. Similarly, the presence of the GST-fusion is unlikely to be interfering with the STK61 protein function, since the GST protein has generally not been seen to interfere with protein function in fusion proteins.

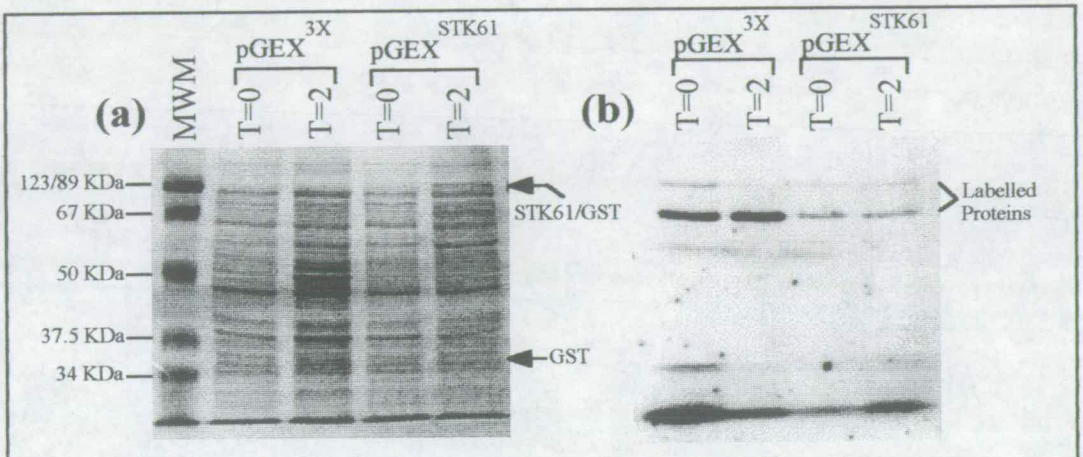


Figure 5.10
Typical result from preliminary kinase assay experiments. (a) SDS PAGE of protein extracts from $pGEX^{3X}$ and $pGEX^{STK61}$ transformed cells, either un-induced (T=0) or induced (T=2). (b) An aliquot of the protein extracts shown in (a) was incubated with γ-radiolabelled ATP and analysed via SDS PAGE and autoradiography. Bands representing phosphorylated proteins are indicated. No increase in kinase activity is seen in the $pGEX^{STK61}$-transformed cells following IPTG induction. MWM=Molecular Weight Markers.

5.2.2.6 Solubility of STK61/GST protein in bacterial cells.

Figure 5.11 shows the result of a pGEX^{STK61} expression experiment, where soluble and insoluble protein has been separated.

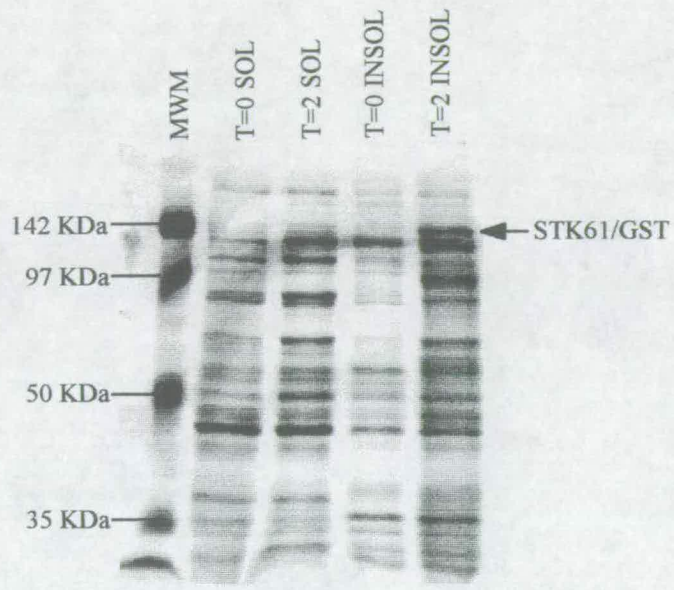


Figure 5.11
SDS PAGE of protein extracts from pGEX^{STK61}-transformed cells, either un-induced (T=0) or induced (T=2) with IPTG. Soluble and insoluble protein fractions were separated and loaded separately. Most STK61/GST fusion protein is insoluble. MWM=Molecular Weight Markers.

It is clear from this that most of the pGEX^{STK61}-expressed protein is present in the insoluble fraction of the bacterial extracts and so is likely to be sequestered in inclusion bodies. Thus, the lack of kinase activity seen from induced pGEX^{STK61} cell extracts does not necessarily indicate that the produced protein is non-functional.

5.2.2.7 Purification of STK61/GST and GST proteins.

Since the STK61/GST fusion protein is largely insoluble in bacterial cells, the use of crude bacterial extracts for functional assays is clearly not adequate. A purification was attempted using glutathione-agarose affinity chromatography to concentrate any fusion protein which may still be in the soluble fraction of the $pGEX^{STK61}$-containing induced cells (figure 5.12).

Figure 5.12
SDS PAGE of various samples taken throughout a GST affinity chromatography protein purification experiment. Glutathione-agarose matrix (GAM) is added to cell lysates. GST protein associates with the glutathione-agarose and is eluted in the presence of reduced glutathione. Sample 1; $pGEX^{3X}$-transformed induced cell lysate, soluble fraction, after binding and removal of GAM. Sample 2; $pGEX^{3X}$-transformed induced cell lysate, soluble fraction. Sample 3; $pGEX^{3X}$-transformed induced cell lysate, after addition of lysosyme and detergent. Sample 4; $pGEX^{3X}$-transformed induced cell lysate, crude extract. Sample 5; $pGEX^{3X}$-transformed un-induced cell lysate, crude extract. Sample 6; $pGEX^{STK61}$-transformed induced cell lysate, soluble fraction, after binding and removal of GAM. Sample 7; $pGEX^{STK61}$-transformed induced cell lysate, soluble fraction. Sample 8; $pGEX^{STK61}$-transformed induced cell lysate, after addition of lysosyme and detergent. Sample 9; $pGEX^{STK61}$-transformed induced cell lysate, crude extract. Sample 10; $pGEX^{STK61}$-transformed un-induced cell lysate, crude extract. Sample 11; Eluted protein from $pGEX^{3X}$-transformed cells (0.2% of total eluate loaded). Sample 12; Eluted protein from $pGEX^{3X}$-transformed cells (0.02% of total eluate loaded). Sample 13; Eluted protein from $pGEX^{STK61}$-transformed cells (15% of total eluate loaded). Some bands are visible in lane 13 which may represent purified STK61/GST protein, but such a large proportion of the total eluate was loaded that these bands may represent carry-over of endogenous bacterial proteins. MWM=Molecular Weight Markers.

Very efficient purification of GST protein from the $pGEX^{3X}$-transformed induced cell control is seen (figure 5.12; lanes 11 & 12). However, very poor recovery is seen from $pGEX^{STK61}$-containing induced cells (figure 5.12; lane 13). This is especially striking considering that around 800 times more glutathione-agarose eluate was loaded in lane 13 than in lane 12. Clearly, virtually no STK61/GST fusion protein remains in the soluble fraction of the $pGEX^{STK61}$-containing induced cells. A lengthy inclusion body purification protocol was not attempted, since this involves denaturation/renaturation of the purified protein and could be deleterious to the protein's function.

In summary, cDNAs a and 11 encode an OPA repeat-containing serine/threonine protein kinase. This protein is produced from the ORF *in vivo*. This protein may be regulated in a tissue-specific or developmentally-regulated manner.

5.3 DISCUSSION

Over recent years a great deal of work has been done on protein kinases which has revealed their importance in developmentally significant processes such as intercellular signal transduction and cell cycle regulation. To further understand what roles a sex-specific kinase might play, it is necessary to review the known functions of kinases in *Drosophila.*

Protein kinases can be divided into two groups depending on which amino acids they catalyse the transfer of phosphate to. These are the tyrosine kinases and the serine/threonine kinases. Tyrosine kinases can be further subdivided into those proteins which are membrane localised receptors (receptor tyrosine kinases, or RTK's) and those which are free in the cytoplasm. The receptor tyrosine kinases can be further subdivided according to the type of intracellular and extracellular domains they possess. The intracellular kinase domain may or may not be split by a kinase insert (KI) region. Extracellularly, the protein may exhibit a number of ligand binding domains such as cysteine repeats, immunoglobulin C2 domains, acidic domains, fibronectin type III repeats and the *sevenless*-like YWTD amino acid repeats. Serine/threonine kinases on the other hand tend to be purely cytoplasmic (reviewed in Pawson & Bernstein, 1990; Seigfried *et al.*, 1990).

5.3.1 PROTEIN KINASES IN SIGNAL TRANSDUCTION

Perhaps the most widespread function of protein kinases is the transduction of intercellular signals into intracellular changes in gene expression. In all cases, this involves an extracellular ligand making itself known to some component of the plasma membrane which then causes intracellular changes to take effect. This can occur in several ways, all of which involve the action of intracellular protein kinases (reviewed in Watson *et al.*, 1987). Some ligands act via the membrane-bound enzymes phospholipase C and adenylate cyclase. When active, phospholipase C catalyses the breakdown of the plasma membrane phospholipid phosphatidyl inositol

diphosphate (PIP_2) into the second messengers inositol-3-phosphate (IP_3) and diacylglycerol (DAG). Adenylate cyclase catalyses the formation of the second messenger cyclicAMP (cAMP) from ATP. Increases in the intracellular concentration of these second messengers activates the cAMP-dependent, Ca^{2+}/calmodulin-dependent and phospholipid/calmodulin-dependent protein kinases which then go on to modulate gene expression. How these kinases exert their function is, as yet, poorly understood. The activity of the enzymes phospholipase C and adenylate cyclase is regulated by the binding of extracellular peptide growth hormones to receptor tyrosine kinases (RTK's). It has been known for some time that tripartite GTP-binding protein (G-protein) complexes (consisting of α, β and γ subunits) are involved in the transduction of extracellular hormone signals into changes in intracellular concentration of second messengers (Gilman, 1984). In the absence of hormone stimulation, GDP is bound to the G-protein complex which is inactive. When the G-protein complex encounters a hormone-bound RTK, the GDP dissociates and is replaced by GTP which activates the complex. The GTP-bound α-subunit then dissociates from the complex and either stimulates or represses the second messenger-producing enzymes. Figure 5.13 shows a summary of these transduction mechanisms.

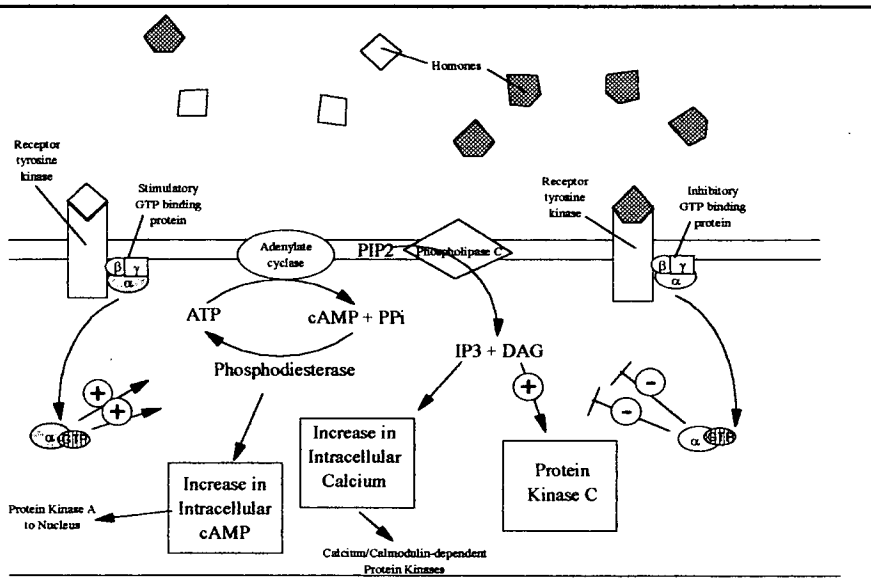


Figure 5.13
Summary of the major pathways of extracellular signal transduction. See text for details.

5.3.1.1 Ras-mediated signaling pathways.

One particular α-subunit G-protein encoded by the *ras* oncogene has recently provided a great deal of information concerning the role of protein kinases in signal transduction. This gene was originally found in a mutated form, causing malignant transformation of mammalian culture cells (Satoh *et al.*, 1992). Like other α-subunit G-proteins, active Ras binds GTP which it gradually hydrolyses to GDP via its own GTPase activity. Furthermore, Ras deactivator proteins, GAP proteins (GTPase Stimulating Proteins), exist which can associate with Ras and stimulate its GTPase activity. Injection of anti-Ras antibody into fibroblast cells suppresses those cells' normal responses to peptide hormones such as PDGF and EGF (Mulcahy *et al.*, 1985), indicating that Ras has a role in transducing extracellular signals via RTK's.

When extracellular ligands bind to RTK's on the surface of the cell, this brings about dimerisation of the receptors which become autophosphorylated on certain tyrosine residues of their intracellular domains (Shilo, 1992). Recently, the mechanisms by which RTK's bring about changes in Ras activity has been further elucidated by studies on a conserved 100 amino acid domain which appears to mediate specific binding to phosphorylated tyrosine residues present in the intracellular domains of RTK's. This domain has been termed the *src* homology-2 (SH2) domain and is found in many signaling proteins including Src, Abl, Phopholipase C and Phosphatidyl inositol (3') kinase (Pawson & Gish, 1992). It has been shown that SH2 domains directly recognise and bind to the phosphotyrosine residues present in the intracellular domain of activated RTK's and that the affinity of these interactions is dependent upon the amino acids immediately surrounding the phosphorylated group. SH2 domain-containing proteins also often contain SH3 domains which have been implicated in binding to proline-rich target sequences in other proteins (Cicchetti *et al.*, 1992). For these reasons, proteins which contain both SH2 and SH3 domains have been postulated to act as adaptor proteins, linking activated RTK's to other components of the transduction machinery (Pawson & Gish, 1992).

Direct confirmation of the function of SH2/SH3-containing proteins as RTK adaptors has come from the cloning and analysis of the mouse *grb2* gene (Suen *et al.*, 1993). The Grb2 protein contains no catalytic domains, consisting of only one SH2 and two SH3 domains. Treatment of culture cells with peptide hormones results in a SH2-dependent association of Grb2 with the cognate receptor tyrosine kinase. The SH3 domains of Grb2 were shown to bind to a different subset of proteins from those bound by the SH2 domain, supporting a function for Grb2 as an adaptor between activated RTK's and other signal transduction mediators.

In a similar way to Ras, injection of anti-Grb2 antibody into mammalian culture cells suppresses these cells responses to PDGF and EGF (Matuoka *et al.*, 1993). This strongly suggests that the Grb2 SH2/SH3 adaptor acts in some way to link activated RTK's to the activation of Ras protein.

The missing link between the Grb2 adaptor and Ras has been revealed by studies on eye development in *Drosophila.* In *Drosophila*, the compound eye is made up of around 800 ommatidia, each consisting of 8 photoreceptor cells (R1-R8) surrounded by 4 non-neural cone cells and 8 accessory cells. The R7 photoreceptor is particularly distinctive, and has been the object of much genetic study (reviewed in Rubin, 1991). Mosaic analysis has shown that while a receptor tyrosine kinase encoded by the *sevenless* (*sev*) gene is required in the R7 cell for correct development, the putative transmembrane protein product of the gene *bride of sevenless* (*boss*) is required in cell R8. Antibody *in situ* evidence shows that the Boss protein is taken into the R7 cell and that this is dependent upon Sev function. This suggests that the Boss protein is in fact the ligand which activates Sev. Genes downstream of the Sev RTK have been identified via a *sev* hypermorphic constitutive allele. These genes include *Son of sevenless* (*Sos*; Rogge *et al.*, 1991), *gap1* (Gaul *et al.*, 1992) and *ras1*, the *Drosophila* homolog of mammalian *ras* (Simon *et al.*, 1991). The cloning and characterisation of another gene important in R7 specification, *downstream of receptor kinases* (*drk*), has enabled the construction of a model for *ras* activation (Olivier *et al.*, 1993). The *drk* gene is highly

homologous to mammalian *grb2*, encoding a protein consisting of one SH2 domain and two SH3 domains. As expected, Drk protein is seen to associate with the plasma membrane in a SH2-dependent manner. *In vitro*, Drk protein binds to an area of the Sos protein known to contain proline rich repeats. Since the *Sos* gene encodes a putative guanine nucleotide-releasing factor, this suggests a model for *ras* activation whereby SH2/SH3 adaptor proteins create a link between activated RTK's and guanine nucleotide-releasing factors which act to release GDP from inactive Ras protein, allowing it to bind GTP and become active. This model is summarised in figure 5.14.

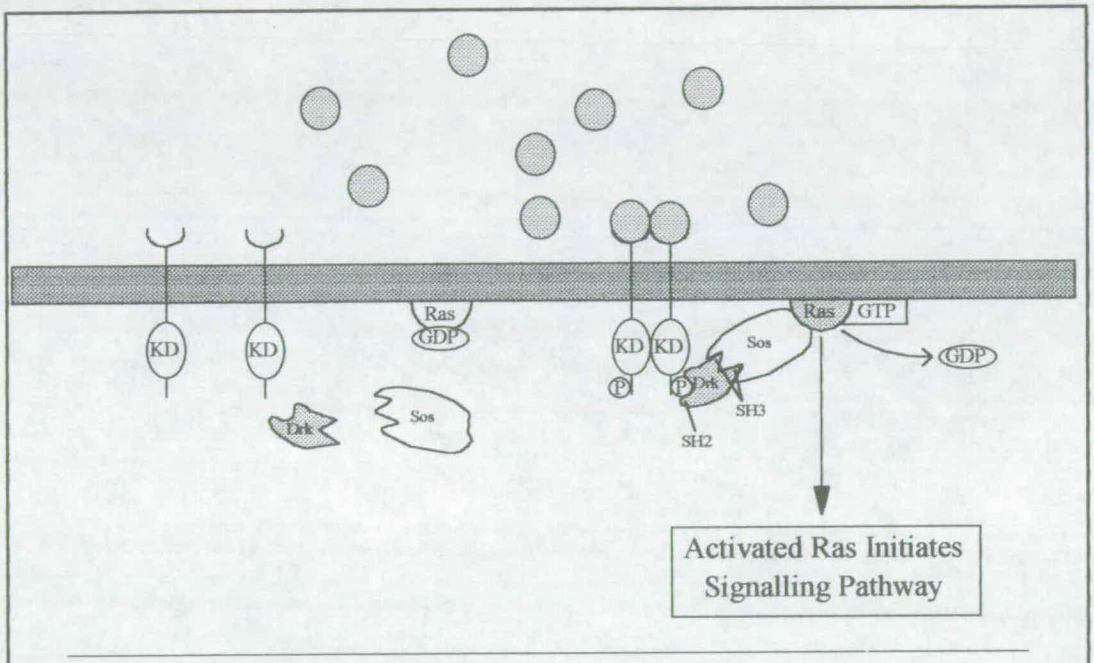


Figure 5.14
Diagram showing model for Ras activation. Extracellular ligands bind to receptor tyrosine kinases, which dimerise and autophosphorylate in their intracellular domains. The SH2 domain of the Drk/Grb2 adaptor protein binds to the phosphotyrosine in the intercellular domain of the receptors. The Guanine nucleotide releasing factor, Sos, contains proline-rich motifs which bind to the SH2 domains of Drk. Sos catalyses the removal of GDP from Ras, enabling Ras to bind GTP and become active. KD=Kinase Domain.

In an attempt to identify the downstream targets of *ras*, active GTP-bound Ras protein was added to mammalian cell culture lysates and then immunoprecipitated using anti-Ras antibody (Koide *et al.*, 1993). The S/T kinase Raf was co-precipitated with Ras, indicating that Raf is a downstream target of Ras with which it physically

interacts. Indeed, direct protein-protein interaction between mammalian Ras and Raf has been demonstrated using the yeast dihybrid screening system (Vojtek *et al.*, 1994). It is clear that Ras activation leads to the subsequent activation of a number of downstream protein kinases, including Raf, which are themselves regulated by phosphorylation (Blenis, 1993). Of these proteins, the mitogen-activated protein kinases (MAP kinases) and the cell-cycle regulated S6 protein kinase (RSK kinase) are particularly interesting. The RSK kinase is a cell cycle-regulated protein which brings about phosphorylation of ribosomal protein, presumably playing some role in cell cycle control. RSK kinase is activated by phosphorylation under control of the S/T-specific MAP kinase. Activation of MAP kinases has been shown to result in the phosphorylation of the Myc and Jun transcription factors. MAP kinase is also activated by phosphorylation, this time under control of the S/T/Y-specific kinase MEK. MEK kinase is also activated by phosphorylation. *In vitro*, Raf kinase is capable of reactivating phosphatase-treated MEK kinase (Howe *et al.*, 1992). Raf kinase also appears to be upstream of MAP kinase (Howe *et al.*, 1992; Samuels *et al.*, 1993). These results suggest a pathway whereby the G-protein Ras activates the S/T kinase Raf, which goes on to initiate a cascade of kinase activity which terminates in phosphorylation of ribosomal proteins and nuclear factors such as transcription factors.

Another level of regulation of Ras-mediated transduction involves the phosphatase, protein phosphatase 2A (PP2A). In *Drosophila*, use of transgenic flies which constitutively express Ras or Raf has revealed that PP2A has a role in regulating the activity of these proteins (Wassarman *et al.*, 1996). Constitutive expression of *ras* results in a number of ommatidial cone cells developing as R7 photoreceptors. If only one functional copy of *PP2A* is present, an increase in these transformations is seen, indicating that PP2A represses members of the Ras-induced pathway. However, a reduction in transformed cells is seen in flies constitutively expressing *raf*, when only one active copy of *PP2A* is present, suggesting that PP2A activates members of the Raf-induced pathway. Thus, it seems clear that PP2A acts on different members of the Sev signaling pathway, as both an activator and a

deactivator. The substrates of PP2A in the Sev pathway are unclear as yet. It may be that multiple forms of PP2A are involved and that some PP2A substrates are yet to be discovered. In a screen for dominant suppressors/enhancers of eye phenotype caused by a hypermorphic *ras* allele, three genes were identified which are involved in the Sev signaling pathway (Wassarman *et al.*, 1996). These are *kinase suppressor of ras* (*ksr*), the product of which acts either upstream of, or in parallel to Raf; *phyllopod* (*phyl*), which encodes a nuclear protein implicated in neural differentiation and *yan*, a transcription factor which represses photoreceptor development.

One particularly striking feature of the RTK–>Ras–>Raf–>MEK–>MAP kinase cascade is the evolutionary conservation of this pathway. Homologs are present in signal transduction pathways from yeast, *C. elegans*, *Drosophila* and mammalian cells, as shown in table 5.1 (see Feig, 1993; Egan & Weinberg, 1993; Crews & Erikson, 1993; Pelech, 1993 for reviews).

ORGANISM	*Drosophila*	*Drosophila*	Mammals	*C. elegans*
RTK	Sev	Torso	PDGF	Let-23
ADAPTOR	Drk		Grb2	Sem-5
PTP		Csw	SH-PTP2	
GRF	Sos	Sos	Sos-1, 2	
GAP	GAP-1		Ras GAP	
G-PROTEIN	Ras1	Ras1	$p21^{ras1}$	Let-60
S/T KINASE	D-Raf	D-Raf	Raf-1	Lin-45
Y/T KINASE		D-Sor1	MEK	
S/T KINASE	Rolled		MAP	
NUCLEAR EFFECTORS?	Sina	Tailless Huckebein		

Table 5.1
Conservation of proteins involved in Ras-mediated signaling. PTP=Protein Tyrosine Phospatase, GRF=Guanine-nucleotide Releasing Factor, GAP=GTPase Activating Protein.

In *Drosophila*, the proteins of the MAP kinase cascade are conserved in at least two systems; the development of the R7 photoreceptor and the determination of the terminal poles of the developing embryo. As we have already discussed, determination of the R7 photoreceptor begins with a signal from Boss to the RTK Sev. The SH2/SH3 adaptor protein Drk acts to couple the activated Sev RTK to the guanine nucleotide releasing factor Sos, which brings about activation of Ras. This is antagonised by the GTPase-stimulating protein GAP-1. Ras activation leads to phosphorylation (by some unknown kinase) of the Raf S/T kinase which, in turn, leads to phosphorylation of the *Drosophila* MEK kinase homolog, Rolled. Mutation of the gene *seven in absentia* (*sina*) results in a similar phenotype to *sev* and *boss* alleles (Carthew & Rubin, 1990). This gene encodes a nuclear putative transcription factor and may be one of the terminal targets of the Sev signaling pathway. The *seven-up* (*svp*) gene encodes a protein with homology to the steroid receptor family, is expressed in photoreceptors R1, R3, R4 & R6, and missexpression of *svp* causes R7 cells to develop as the photoreceptors which usually express *svp* (Hiromi *et al.*, 1993). Thus, Svp may act to repress the effects of the Sev pathway in non-R7 photoreceptors.

The terminal effect genes are involved in determining the positional identity of the extreme ends of the developing embryo and have been discussed in detail in Chapter 1. The RTK Torso (Tor) is thought to be activated at the ends of the embryo be the extracellular ligand Torsolike (Tsl). Downstream of the Tor RTK, the product of the gene *l(1)pole-hole* (*l(1)ph*) regulates the expression domains of the gap genes *tailless* and *huckebein* which are responsible for the positional identity of the embryo's terminal structures (Johnston & Nüsslein-Volhard, 1992). The gene *l(1)ph* in fact encodes the *Drosophila* homolog of *raf* which is clearly important in the determination of embryonic terminal structures, as well as in specification of the R7 photoreceptor (Melnick *et al.*, 1993). Indeed, the Tor RTK signaling pathway involves *Drosophila* homologs of *ras* (*ras1*), *PP2A* (*corkscrew*), MAP kinase (*rolled*) and MEK kinase (*D-sor1*), as well as the Sos SH2/SH3 adaptor protein (Perrimon, 1993). Recent evidence using germline mosaic flies, mutant for different

genes in the Tor RTK-mediated pathway, suggests that the Raf-MAP kinase cascade can also be activated from Tor in a separate pathway which is independent of Drk, Sos and Ras (Hou *et al.*, 1995).

In general, it appears that the Ras-mediated signaling pathway is brought into play by a number of different RTK's which effect different changes within the cell (Feig, 1993). The fact that different RTK's can bring about different functions while still using the same set of highly conserved kinases presents something of a paradox. We might expect a sex specifically-expressed kinase, such as STK61, to be involved in modulating the effects of the Raf-MAP kinase cascade in a sex-specific way. Indeed, there may be a whole range of sex-specific and tissue-specific factors which are expressed in precisely defined areas which function to "funnel" the general driving force of the MAP kinase cascade down very specific pathways. The fact that STK61 contains OPA repeats, which are found in proteins encoded by genes with clearly defined expression patterns, suggests that this protein may be involved in such a tissue-specific function.

In fibroblasts, increasing the levels of cAMP leads to an inhibition of Raf activation by peptide hormone stimulation (Burgering *et al.*, 1993). This indicates that an interplay between Ras-mediated and second messenger-mediated signaling can occur. Indeed, it is most likely that transduction of a particular signal to produce a specific effect involves a number of different pathways which act together to result in the appropriate response.

5.3.2 PROTEIN KINASES IN CELL CYCLE REGULATION

The oncogenic effects of hypermorphic alleles of genes involved in signal transduction pathways shows that a major purpose of the transduction of extracellular signals is to bring about changes in the cell division cycle. Indeed, the yeast cell cycle-regulatory phosphatase CDC25 was the first guanine nucleotide exchange factor identified in the Ras signaling pathway. A vast amount of work has

been done on the molecular control of the cell cycle and a simplified model derived from the obtained results is shown in figure 5.15 (reproduced from figure 1.6).

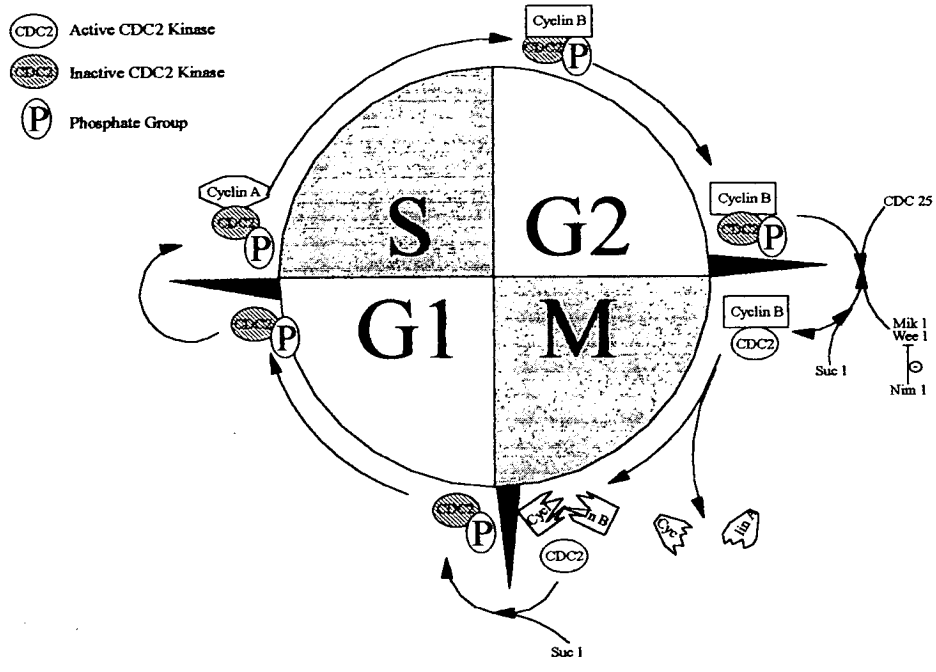


Figure 5.15
Diagram showing the major gene products involved in cell division cycle regulation (reviewed in Pines & Hunter, 1990; Woodgett, 1991; Enoch & Nurse, 1991; MacNeill *et al.*, 1991; Glover, 1991). A great deal of this work was carried out on the fission yeast *S. pombe*, but homologous genes have been found in a number of organisms, including humans, and have been shown to be functionally interchangeable in a number of cases. S=S-phase (DNA synthesis phases), M=M-phase (Mitotic division phase), G1=Gap-phase 1, G2=Gap-phase 2.

The CDC2/p34 Serine/Threonine (S/T) kinase is thought to be the main 'workhorse' of cell cycle regulation, acting to drive the cell through the divisions between the phases of the mitotic cycle, dependent upon its phosphorylation state and association with the cell cycle-regulated Cyclin proteins. Dephosphorylation of CDC2 in the CDC2/CyclinB complex in G2-phase enables transition from G2 into M-phase. Dephosphorylation of CDC2 is brought about by the CDC2-activator, CDC25 phosphatase. The Wee1 protein (and its redundant homologue Mik1) is a Serine/Threonine/Tyrosine (S/T/Y) kinase which inhibits cell cycle progression and may phosphorylate CDC2. The Nim1 S/T kinase is thought to negatively regulate Wee1 via phosphorylation. Suc1 protein physically associates with CDC2 and regulates G2/M transition (negatively) and mitotic progression (positively).

Not only does the entire cycle depend upon the phosphorylation state of a protein, but this protein is itself a kinase; the CDC2 kinase. The targets of active CDC2 kinase include all manner of proteins which are required for the process of M-phase, such as nuclear lamins (for nuclear breakdown/reassembly), histone H1 (for

chromatin decondensation), the HMG proteins (involved in heterochromatin formation) and the myosin light chain (involved in spindle operation). The proteins involved in cell cycle regulation (including the kinases) are ubiquitous, with homologs in organisms as diverse as *Xenopus*, *Drosophila* and yeast. These proteins have been shown to be functionally interchangeable in a number of cases (MacNeill *et al.*, 1991; Pines & Hunter, 1990; Nurse, 1990).

In *Drosophila* embryogenesis there is a particularly interesting example of cell cycle regulation (reviewed by Glover, 1991; Enoch & Nurse, 1991). After the male and female pronuclei fuse in the embryo, the first 10 nuclear divisions are the most rapid in the animal kingdom. At this stage there is no G1 or G2 phases but just rapid S-M cycling. The *giant nuclei* (*gnu*) gene is amongst a number of genes, including the cell cycle-regulated genes *polo*, *mh* and *fs(1)Ya*, which supply protein maternally to the syncitial blastoderm. Embryos from mothers mutant for the *polo* gene have disrupted early divisions with spindles not forming correctly. *mh* and *fs(1)Ya* mutations have the effect of preventing male and female pronuclear fusion which can result in the production of haploid nuclei in the embryo. Females mutant for *gnu* produce syncitial blastoderm embryos containing nuclei that cannot enter M-phase. However, these nuclei do not stall but go through repeated S-phase cycles, giving rise to giant nuclei. This indicates that, at this stage of development, the S and M phases are uncoupled, with entry into M-phase being independent of completion of S-phase. In agreement with this idea of uncoupling, treatment of the embryo with the DNA synthesis inhibitor aphidicolin results in nuclei that go through repeated M-phases without S-phases. This uncoupling may be due to the naturally high levels of maternally supplied String/CDC25 phosphatase present in the embryo, acting to drive CDC2 kinase into M-phase irrespective of the status of S-phase.

At nuclear division 14, when cellularisation has taken place, G2 phase begins and S-phase becomes coupled to M-phase. G1 phase doesn't enter the cycle until division 17. Embryos which are mutant for *string* stall in G2 at division 14, since maternally-supplied String is degraded by this time. Short pulses of ectopic

String/CDC25 phosphatase from a P-element construct, controlled by a heat shock promoter, can allow morphogenesis to occur fairly normally until mid embryogenesis, when death occurs (Edgar & O'Farrell, 1990). Thus, at division 14 the regulation of the cell cycle is brought under zygotic, rather than maternal, control. In wild type embryos, phosphorylated CDC2 kinase (inactive) accumulates during G2, awaiting the production of zygotic String/CDC25 phosphatase which will activate it and drive the cells into S-phase (Edgar *et al.*, 1994).

As well as it's functions in signal transduction, the PP2A phosphatase is also required for cell cycle regulation, with *Drosophila PP2A* mutants showing defects in certain aspects of anaphase (Mayer-Jaekel *et al.*, 1993). It is thought that the PP2A phosphatase may act in a similar way to String/CDC25 phosphatase, activating CDC2 kinase, or may regulate the activity of String/CDC25 phosphatase itself.

The cell cycle-regulatory genes are equally important in the male gonad, where they are required to direct the cell cycle during meiosis, although less is known about exactly what factors are regulating the cell cycle in this tissue. In contrast to the case of oocytes, spermatocytes do not contain large quantities of parentally-derived String/CDC25 phosphatase, and a second CDC25-like phosphatase, the Twine/CDC25 phosphatase, has been shown to be required for CDC2 kinase activation in this tissue (Sigrist *et al.*, 1995). Both male and female *Drosophila*, mutant for *twine*, exhibit meiotic defects which lead to sterility. It appears that the String/CDC25 phosphatase is able to compensate for loss of Twine/CDC25 phosphatase in oocytes since some meioses do occur. In males, however, neither meiotic division occurs in *twine* mutants. This evidence suggests that meiosis in the testis may require gene products which are not required for cell cycle regulation in other tissues.

5.3.3 PROTEIN KINASES IN MAINTENANCE OF SEGMENT POLARITY

S/T kinases are also involved in the differentiation of segment boundaries in *Drosophila*. The genes which set up the parasegments in the developing embryo are called the segmentation genes. Figure 5.16 summarises the roles which have been proposed for these genes in maintaining segment boundaries through development.

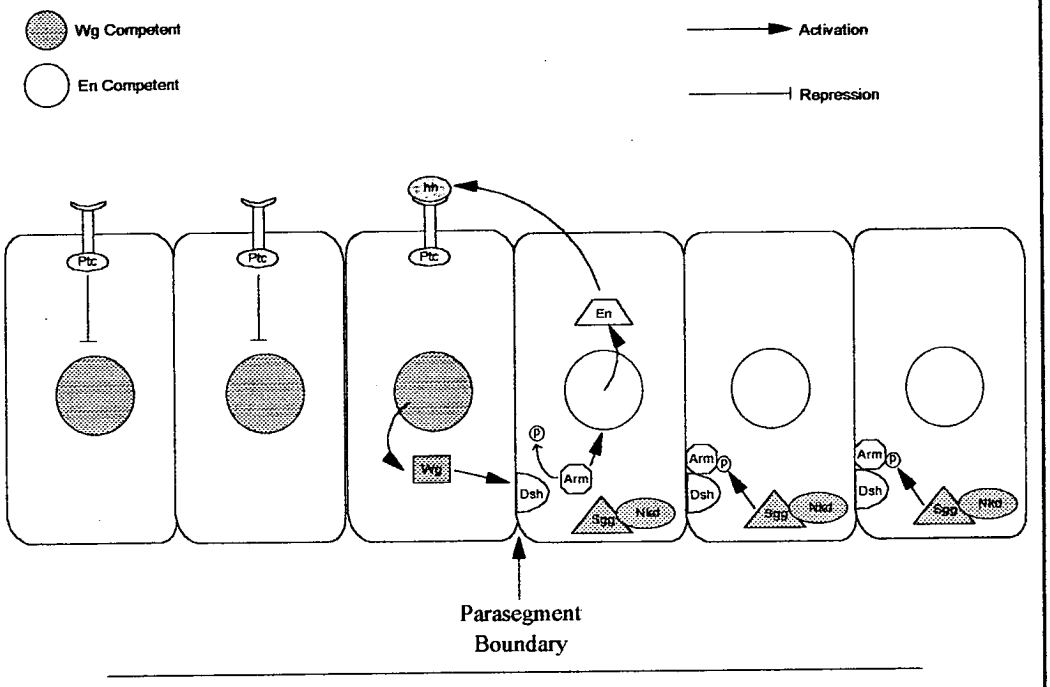


Figure 5.16
Diagram showing model for functions of segment polarity genes (Arias *et al.*, 1988; Ingham, 1991; see main text for further details). In the early embryo, differential concentrations of the products of the *fushi-terazzu* and *even-skipped* pair-rule genes determines cells to be 'competent' to express either *wingless* (*wg*) or *engrailed* (*en*). Thus, *wg*-competent and *en*-competent bands of cells alternate along the length of the embryo. However, Wg and En proteins are expressed only in the cells bounding the parasegment boundary, maintaining the positional identity of cells within the parasegment. This localised expression pattern is maintained as shown above. The *wg* gene is repressed via the Patched (Ptc) receptor. Expression of *hedgehog* (*hh*) occurs only in *en*-expressing cells and alleviates Ptc-mediated repression. The *en* gene may be activated via the Armadillo (Arm) protein, which relocalises from the plasma membrane to the cytoplasm under the action of Wg signaling. The *dishevelled* (*dsh*) gene also appears to be required for reception of this signal. Naked (Nkd) and Shaggy (Sgg) proteins are believed to prevent Arm function in non-boundary cells. Mutation of the *sgg* gene allows Arm relocalisation in the absence of Wg signal, and Sgg has been shown to phosphorylate Arm. Thus, activation of Arm may occur by dephosphorylation, under the action of Wg signal from neighbouring cells.

Two S/T kinases have been implicated in this process. These kinases are encoded by the genes *fused* (*fu*) and *shaggy* (*sgg*, or *zeste-white 3*). Wild type embryos exhibit clear segmentation, with the anterior portion of the segment containing a denticle belt and the posterior composed of naked cuticle. Embryos carrying mutant alleles of *fu* develop a mirror image of the anterior denticle belt in the posterior of each segment (Nüsslein-Volhard & Weischaus, 1980). Other genes which produce this phenotype are *arm*, *dsh*, *en*, *hh* and *wg*. We can see from figure 5.16 that these last five genes are all thought to be involved in the production or transduction of the *wg* signal which allows *en* expression in the anterior band of each parasegment. Since *wg* expression is not maintained in embryos mutant for *fu* (Limbourg-Bouchon *et al.*, 1991), the Fused S/T kinase is thought to have a role in the transcriptional activation of the *wg* gene. This could either be a direct activation of *wg* transcription, or an indirect modulation, possibly by activating the En protein which would lead to Hh-repression of Ptc, thereby allowing expression of *wg*.

Embryos carrying mutations in the genes *nkd* or *sgg* exhibit the opposite phenotype to that described above, with a mirror image of the posterior naked cuticle developing in the anterior of each parasegment (Nüsslein-Volhard & Weischaus, 1980). As shown in figure 5.16, these genes are believed to be involved in the repression of *en* expression in the absence of *wg*-induced signaling. Both *wg*-induced signaling and mutations of *sgg* result in cytoplasmic accumulation of the Arm protein, which is normally membrane-localised (Peifer *et al.*, 1994a). Additionally, Sgg kinase is capable of phosphorylating Arm protein, and this phosphorylation is suppressed by Wg signaling (Peifer *et al.*, 1994b). This strongly suggests a model whereby the Sgg S/T kinase is responsible for maintaining the phosphorylation state of Arm, until the Wg-signal causes it to be de-phosphorylated, enabling it to dissociate from the plasma membrane and lead to activation of *en* expression.

5.3.4 PROTEIN KINASES IN DORSOVENTRAL POLARITY DETERMINATION

Kinase-mediated signaling is also vital to the establishment of the dorsal-ventral axis of the *Drosophila* embryo. In essence, this system involves the transduction of a signal from the overlying follicle cells which is received by receptors encoded by the gene *toll* on the ventral surface of the embryo. This results in a nuclear gradient of the morphogen, Dorsal protein, with ventral nuclei containing higher levels of this protein (Roth *et al.*, 1989). The downstream targets of the putative transcription factor Dorsal are *zerknült* and *decapentaplegic*, which are activated at low Dorsal concentrations and *twist* and *snail*, which are activated at high Dorsal concentrations (see chapter 1 for details). When Toll is inactive, a S/T kinase encoded by the *pelle* gene is found near the plasma membrane, complexed with a protein of unknown function, Tube, and Dorsal is localised in the cytoplasm in a complex with the protein Cactus. Recent co-transfection assays In *Drosophila* culture cells have suggested that the S/T kinase Pelle is involved in mediating Dorsal nuclear localisation (Norris & Manley, 1996). These studies show that Pelle is capable of enhancing both nuclear localisation and transcriptional activity of Dorsal. It was also shown that these Pelle-mediated effects were enhanced by over-expression of the *toll* gene. The Pelle kinase is capable of phosphorylating the Tube protein *in vitro* (Grosshans *et al.*, 1994). The Tube protein has been shown to be essential for the differentiation of dorsal-ventral polarity (Letsou *et al.*, 1993). Norris & Manley, 1996, showed that Tube could re-localise to the nucleus and that this was dependent upon Toll and Pelle function. Thus, it seems likely that activation of Toll receptor leads to activation of Pelle kinase which phosphorylates Tube causing it to permit dissociation of Dorsal from Cactus and relocalisation of Dorsal to the nucleus.

One of the targets of Dorsal, the *decapentaplegic* (*dpp*) gene, encodes a growth factor with homology to the TGF-β family and is itself involved in kinase-mediated signaling. The S/T receptor kinase encoded by the gene *saxophone* is thought to be a Dpp receptor, mediating it's functions in signaling (Xie *et al.*, 1994). Generally, growth factor ligands of the TGF-β family are specific to receptor kinases which are

serine/threonine-specific, with Dpp acting via a number of receptors of this type (Ruberte *et al.*, 1995).

Another gene which has a role in the differentiation of dorsal-ventral polarity in *Drosophila* is the *torpedo* locus. This gene encodes the *Drosophila* homolog of the mammalian epidermal growth factor RTK. Mutations at this locus produce highly pleiotropic phenotypes, including embryo ventralisation, germ band extension defects and CNS defects (Clifford & Schüpbach, 1992). Since mutations in the genes encoding the Sos protein can suppress or enhance the eye phenotypes of certain *torpedo* alleles, it seems that the Torpedo RTK leads to transduction of signals via a Ras-mediated pathway in at least some cases (Rogge *et al.*, 1991). Alleles of *torpedo* which cause embryo ventralisation are maternal in nature, with embryos from mutant mothers exhibiting the phenotype. Although the precise details of the basis of this phenotype are unclear, the role of Torpedo is believed to be the repression of the Toll pathway, at the dorsal side of the embryo (Pawson & Bernstein, 1990). The current model for this Torpedo function states that a signal is sent to the dorsal follicle cells from the embryo which activates the Torpedo RTK. Active Torpedo then transduces this signal resulting in the production of another signal which is sent to the embryo and acts to repress the Toll pathway. RNA from the genes *K10* and *gurken* is found localised around the dorsal nuclei in the syncitial blastoderm and a *gurken* null mutation results in *torpedo*-like ventralisation. It is possible that these genes are responsible for the initial signal to the overlying follicle cells (Pawson & Bernstein, 1990).

5.3.5 DIRECT SIGNAL TRANSDUCTION VIA PROTEIN KINASES

Thus far we have been discussing kinase-mediated signaling pathways which involve fairly complex interactions between several factors. However, signals can also be transduced from extracellular ligands into nuclear effects by surprisingly direct means. The mammalian transcription factor p91 directly associates with the activated epidermal growth factor RTK via it's SH2 domain (Fu & Zhang, 1993).

This leads to activation of p91 by tyrosine phosphorylation. When activated, p91 re-locates to the nucleus and activates transcription of the *c-fos* gene (Fu & Zhang, 1993). Additionally, a nuclear localisation sequence (NLS) has been identified in several proteins and appears to be sufficient for nuclear relocation of proteins (Whiteside & Goodbourn, 1993). It is possible that there are a number of signal transduction proteins which utilise systems such as these to bring about direct transduction of extracellular signals.

5.3.6 PROTEIN KINASES IN CYTOSKELETAL INTEGRITY

Another interesting S/T kinase has been found which is required for the correct development of the eye in *Drosophila*. The *nina-c* locus encodes two proteins with putative kinase domains joined to myosin heavy chain-like domains suggesting a role involving interaction with cytoskeletal proteins. Mutants at this locus have eye photoreceptors with reduced rhodopsin levels which gives rise to unusual neural responses. This lack of rhodopsin appears to be due to the reduction in size of the rhabdomeres (Membranes containing rhodopsin and other phototransduction molecules) present in these mutants (Seigfried *et al.*, 1990). It is likely that the *nina-c* kinase acts upon cytoskeletal proteins in the cell to maintain the integrity of the rhabdomeres.

5.3.7 NON-RECEPTOR TYROSINE KINASES

Non-receptor tyrosine kinases are also important in *Drosophila* development as indicated by the studies on the *abl* locus (Hoffman, 1991). The *abl* gene encodes a predicted protein with SH2, SH3 and kinase homology. Mutants have fairly mild phenotypes, with hypomorphic alleles allowing flies to develop to adulthood but with irregular, roughened eyes. Even individuals carrying complete null *abl* alleles develop as far as the late pupal stage. It would appear that functional redundance is the cause of the mildness of these phenotypes, as embryos which are also mutant for the genes *disabled, failed axon connections, prospero* and the *fasciclin I* gene

(Fasciclin I is a cell adhesion molecule produced in the CNS at the same time as *abl* when axon connections are forming) have much more severe phenotypes of the CNS and muscle resulting in late embryonic or early larval death (Hoffmann, 1991). The function of Abl is to some extent independent of its kinase activity, since complete rescue of *abl/abl* mutant flies is achieved by germline transformation of a P-element construct which ectopically expresses kinase-defficient Abl protein. Kinase function is required for other processes, however, since only a construct expressing the active kinase fully rescues the double mutant *abl/abl, disabled/disabled* (Hoffmann, 1991).

5.3.8 PROTEIN KINASES IN NEUROGENESIS

S/T kinases have been shown to be involved in postembryonic differentiation of the *Drosophila* CNS. Studies on cell-cell interactions involving the *Notch* and *Delta* neurogenic loci have provided a great deal of information regarding the mechanisms of early embryonic neurogenesis (for reviews see Vaessin *et al.*, 1990; Artavanis-Tsakonas & Simpson, 1991). However, much less is known about which genes are responsible for directing later postembryonic differentiation of the CNS. Mutant alleles of the *minibrain* (*mnb*) gene result in a reduction in size of both male and female brain, with female brains showing slightly more reduction than males (Tejedor *et al.*, 1995). The overall architecture of brains from *mnb* mutant flies seems to be fairly well preserved, although the optic lobes are disproportionally reduced in size and some neuron loss is seen. As might be expected, *mnb* adults exhibit some behavioural abnormalities, including loss of attraction to a vertical stripe, and loss of odour discrimination. Courtship behaviour has not been examined. The *mnb* gene encodes a S/T kinase with homology to cell cycle regulatory kinases and the *mnb* phenotype seems to result from a reduction in the number of progeny cells produced from certain neuroblasts. This suggests that the Mnb kinases may be involved in regulating neuron-specific cell division.

The analysis of the *mnb* gene has interesting implications with regard to *stk61*. Clearly, male and female *Drosophila* exhibit sex-specific behaviours which must

have a foundation in CNS differences. Indeed, the CNS structure is sexually dimorphic, with females having larger numbers of Kenyon fibres and males exhibiting extra divisions of the terminal abdominal neuroblasts (Technau, 1984). These extra male neuroblast divisions are dependent upon the *dsx* gene, however, making it unlikely that *stk61* has a significant role to play in this particular process. RNA *in situ* evidence from our lab suggests that *stk61* may be expressed in the brain of third instar larvae (D. Clyde, pers. comm.), although it is not known whether fully processed transcript is present or if active protein is produced in this tissue. However, *stk61* may have some role in neuroblast regulation, influencing the differentiation of subtle sexually dimorphic CNS characteristics.

5.3.9 CONCLUSIONS

There are examples of the requirement for kinases in every major developmental process, from the gradual restriction of totipotency (as in the cases of *fu* and *sgg*) to terminal differentiation (as in the action of *nina-c*). Based upon these observations, some specific predictions were made concerning possible functions of the OPA repeat-containing S/T kinase encoded by the *stk61* gene.

In general, it is not hard to imagine how S/T kinase-mediated processes such as signal transduction and cell cycle control could contribute to the establishment of sexually dimorphic characteristics. Indeed, the ubiquitous nature of protein kinase-regulated pathways in development makes it reasonable to assume that sex differentiation processes will involve these proteins. However, since the *yp* genes remain the only cloned sex differentiation genes, a role for protein kinases in sex differentiation is still to be demonstrated. For this reason, we hope that studies on the sex-specifically regulated S/T kinase gene, *stk61*, will provide an inroad into the regulatory processes required for differentiation of the sexually dimorphic features of *Drosophila*.

In *Drosophila* there are many processes in which a role for a sex-specifically expressed S/T kinase, such as that encoded by *stk61*, could be envisaged. Clearly, initial rounds of nuclear division in the syncitial blastoderm during embryogenesis requires close control of the cell division cycle. The ovary-specific 3.5kb *stk61* transcript may express STK61 protein in this tissue which could certainly play a part in this process. Similarly, in the developing female embryo an inductive signal from the somatic component of the gonad is required for the germ cells to be determined as female (see chapter 1 for details). Again, an ovary-specific kinase may be required to allow female germ cells to respond to this signal. Control of the cell division cycle is equally important in male germ cell development, where a testis-specific kinase (possibly encoded by the testis-specific 3.0kb *stk61* transcript) may come into play. Close control of the cell division cycle, as well as modulation of positional information, would also be required to bring about the sex-specific structures of male and female *Drosophila* abdominal tissue. The fact that STK61 contains OPA repeats indicates that it may be localised to a specific tissue or area in the female body, where it may play the part of a sex-specific "filter" of extracellular signals, allowing signals to be interpreted in a female-specific way. This could be a developmental role, where *stk61* may act to define a specific group of cells as female, thereby influencing their development. Alternatively, *stk61* may be active in the adult fly, perhaps acting to transduce pheromonal, or other sex-specific signals. In the next chapter I will discuss what strategies might shed light on the functions of the *stk61* gene.

CHAPTER 6

FINAL DISCUSSION

6.1 SUMMARY OF RESULTS

Chapters 3, 4 and 5 describe the cloning and characterisation of a gene which we have named *Serine/Threonine Kinase 61* (*stk61*). This gene was isolated via a sex-specific differential screen designed to detect genes which produce non-gonadal sex-specific transcripts. The *stk61* gene produces several sex-specific transcripts, including a 4.5kb female carcass-specific transcript, a 3.5kb ovary-specific transcript and a 3.0kb testis-specific transcript. cDNAs representing the testis-specific and female carcass-specific transcripts were cloned and sequenced. Both cDNAs contain an identical long open reading frame, encoding a OPA-repeat-containing serine/threonine-specific protein kinase. This open reading frame is functionally relevant, since apparently full-length protein can be translated in bacterial cells. Northern blot analysis indicates that production of the fully processed female carcass-specific transcript is dependent upon the *tra* sex determination gene. Consensus sequences known to be required for Tra/Tra-2 regulation of *dsx* transcripts have been delimited in the sequence of the *stk61* cDNAs. Thus, the female carcass-specific transcript is likely to be under direct control of the Tra and Tra-2 proteins. The cDNA representing the testis-specific transcript contains within its 5'UTR two copies of a male germline-specific translational control element (TCE) which mediates translational repression until late in spermiogenesis.

6.2 IMPLICATIONS OF RESULTS AND FUTURE WORK

6.2.1 FEMALE CARCASS-SPECIFIC TRANSCRIPT

The apparent mode of regulation of the female carcass-specific transcript suggests that the *stk61* gene may be involved in sex-specific differentiation of non-gonadal tissue.

Mutations in the *tra* or *tra-2* genes result in female somatic development being subverted to the male pathway, with *XX/tra* or *XX/tra-2* flies developing as pseudo-males. Since the complete splicing of the female carcass-specific *stk61* transcript appears to be under direct control of the *tra* gene product, it is reasonable to assume that this *stk61* transcript produces a protein which is involved in transducing the 'ON' state of the *tra* gene into female-specific development. This is of particular interest because the majority of sex-specific features which are under *tra/tra-2* control represent not a direct dependence upon *tra* or *tra-2*, but rather a dependence upon the *dsx* gene, which is regulated by *tra* and *tra-2*.

Certain aspects of male courtship behaviour have been hypothesised to be repressed directly by Tra and Tra-2 (see chapter 1 for details). Since *stk61* encodes a S/T-specific protein kinase, it is likely that STK61 protein is involved in production or reception of an extracellular signal of some kind. A possible role for *stk61* in reception of sex-specific behavioural signals (e.g. pheromones), or modulation of global signals in a sex-specific way, cannot be ruled out. There are two sex-specific processes in *Drosophila* which are known to require intercellular signaling. The first of these is the differentiation of the male-specific muscle of Lawrence (MOL) in the fifth abdominal segment. In this case, both *tra* and *tra-2* genes are required to prevent development of the muscle, but *dsx* is not. Since the determining factor seems to be the sex of the MOL innervating axons, rather than the sex of the MOL itself, *tra* and *tra-2* must be having their effect in these axons. Clearly, a signal must be sent from the innervating axons to the muscle precursor cells, in the developing

embryo, which either initiates or represses the development of the MOL. In the segment polarity system (see figure 5.16), the Shaggy S/T kinase is directly involved in preventing production of a signal which acts upon neighbouring cells. In the same way, the STK61 S/T kinase may act in the female MOL-innervating axons to prevent production of a MOL-inducing signal. The presence of OPA glutamine repeats in the STK61 protein suggests that the protein is localised to a specific tissue or subset of cells. At present, work leading to the production of STK61 antibodies is underway in the lab, which will enable the expression pattern of the protein to be determined. It may be that STK61 protein will be found to be localised to certain PNS axons, which would support a role for *stk61* in MOL repression. As discussed previously, another OPA-containing protein, the Notch protein, is known to function in early neurogenesis. This lends further weight to a possible neurogenic role for STK61.

6.2.2 TESTIS-SPECIFIC TRANSCRIPT

At present it is unclear from the available whole mount RNA *in situ* hybridisation data whether *stk61* RNA is localised to the somatic or to the germline component of the testis. However, the presence of TCE elements in the 5' UTR of the testis-specific transcript strongly suggests that STK61 protein functions in the male germline. Precise cell-division-cycle regulation is vital in development of spermatocytes and a number of protein kinases which are involved in spermatocyte cell cycle regulation have already been discussed. However, it is unlikely that STK61 kinase is involved in this process in the male germline, since TCE-containing transcripts only become translationally active late in the elongation phase of spermiogenesis, when haploidisation has already occurred. It is much more likely that STK61 plays some part in the later processes which lead to morphological differentiation of the spermatozoon. This may involve transduction of signals sent from the somatic component of the testis, or even regulation of the integrity of cytoskeletal structures (as with the Nina-C kinase). Again, determination of the precise expression pattern of testis-specific STK61, via anti-STK61 antibody *in situ* analysis, will hopefully provide some clues as to the role of the protein in this tissue. Similarly, antibody *in*

situ analysis could be used to examine the function of the TCE elements present in the testis-specific transcript. No germ cells beyond the primary spermatocyte stage are found in 3rd instar larvae. As with the *Mst(3)CGP* gene family, we would not expect to find STK61 protein in these cells, unless the TCE sequences are mutated.

6.2.3 OVARY-SPECIFIC TRANSCRIPT

As yet, we have not isolated a cDNA which represents this transcript. However, Northern blot analysis shows that it does not contain the long 3' UTR sequence which is specific to the female carcass-specific transcript. It does contain sequences covering the open reading frames of both of the other transcripts. It is possible that the ovary-specific transcript contains a 3' UTR sequence which is not found in either of the other two sex-specific transcripts.

The ovary-specific transcript was not detected in XY/tra^{FEM} pseudofemale flies. However, examination of the gonads of the pseudofemales used for this analysis showed that they were not well transformed towards female-like ovaries, still containing accessory gland-like structures (figure 4.4). More complete transformation to 'pseudo-ovaries' can be achieved by heat-shock which increases production of ectopic Tra in flies carrying the tra^{FEM} construct. We would expect such pseudo-ovaries to contain the ovary-specific *stk61* transcript, only if it is expressed in the somatic component of the ovary.

Determination of the precise localisation and sequence of this transcript is a high priority for future work. If the *stk61* ovary-specific transcript is expressed in the somatic component of the ovary, it would be reasonable to suppose that this transcript would be under the direct control of the *tra* gene product, in the same way as is proposed for the female carcass-specific transcript. This is particularly interesting in the light of evidence which shows that the somatic inductive signal, which is required for female germline differentiation, is under control of *tra-2*, but not *dsx* (see chapter 1 for details). It is attractive to propose that the role of the *stk61*

ovary-specific transcript may be in the production of this signal. However, the *tra* gene does not appear to be required for this signal to be produced. Since the female carcass-specific *stk61* transcript appears to be more dependent upon *tra* than *tra-2* (or, at least, equally dependent), this may not be the function of *stk61* in this tissue. The ovary-specific transcript may, instead, be germline-specific, possibly acting to transduce signals within the oocyte. The elucidation of the functions of this transcript awaits cloning of the cDNA and precise determination of the RNA and protein localisation within the female gonad.

6.2.4 FUTURE PROSPECTS

The work presented here strongly suggests that at least some of the transcripts from the *stk61* gene are regulated directly by Tra/Tra-2 proteins. Clear confirmation of this model by Northern analysis has proved extremely difficult, for reasons discussed earlier. One approach which could prove more effective is RNAase protection analysis. This would involve subcloning a cDNA fragment bounding the putative Tra/Tra-2-regulated splice site into a transcription vector. Thus, a radiolabelled riboprobe could be generated, by *in vitro* transcription. This riboprobe would then be hybridised with RNA extracts from male and female carcass tissue and treated with RNAase, to remove any single-stranded molecules. Analysis of these reactions by polyacrylamide gel electrophoresis and autoradiography would reveal a band diagnostic of fully-spliced *stk61* transcript. If the female carcass-specific transcript is produced by removal of intron 4, as proposed, we would only expect to see this band in reactions performed with female carcass RNA. This same technique could then be used to examine the involvement of *tra* and *tra-2* gene products in removal of intron 4, by performing RNAase protection analysis using RNA extracts from *tra* or *tra-2* mutant flies.

In the longer term, a direct role for Tra and Tra-2 in *stk61* intron 4 removal could be demonstrated by use of *Drosophila* cell culture co-transfection assays. This technique has been used to demonstrate a direct requirement for Tra and Tra-2

proteins in splicing regulation of transcripts from the *dsx* gene (see chapter 1 for details). An expression vector containing *stk61* genomic DNA, encompassing intron 4, would be transfected into culture cells either with or without expression vectors encoding Tra and/or Tra-2 proteins. The *stk61* transcription products could then be analysed using RT-PCR, or RNAase protection assays. If the intron 4 splice acceptor site is indeed regulated by Tra/Tra-2, we would only expect to see this intron removed in cells expressing Tra and Tra-2 proteins.

6.2.4.1 P-Element Mutagenesis.

In order to definitively assign a function to the *stk61* gene, it will be necessary it introduce mutations into the gene. It is possible to interfere with the function of a specific gene in *Drosophila* by introduction of a P-element transposon into the locus. When males with P-elements present in the genome are mated to females lacking P-elements, the P-elements are mobilised in the germ cells of the resulting embryo, 'hopping' to new positions in the genome. The efficiency of this process can be increased by mating flies which have mobilisable transposase-deficient P-elements to flies with un-mobilisable P-elements (Δ2-3 elements) which produce transposase (Zhang & Spradling, 1993). This process is random and there is no way of predicting where the P-elements will relocate. However, use of a P-element-specific oligonucleotide primer and a gene-of-interest-specific primer enables identification of flies in which a P-element has relocated close to a gene of interest, via PCR analysis (Kaiser & Goodwin, 1990). Males of the mobilisable P-element strain are mated to females of the Δ2-3 transposase-producing strain. F1 males are collected and mated to wild-type females to produce true-breeding F2 flies. The eggs from F2 females can be collected and analysed by PCR. One fly with a P-element inserted close to the gene of interest can be detected in a DNA sample representing 1000 flies. By dividing these flies into smaller and smaller groups, re-testing each time, single flies with the P-element insertion can be isolated. Conversely, P-elements which hop from close to the gene of interest can result in deletions in the locus. These can be detected by Southern blot analysis.

6.2.4.2 Germline Transformation Studies.

Another approach which can provide information on the function of a gene is to mis-express the gene ectopically in inappropriate tissues, or in flies of the inappropriate sex. This can be done in *Drosophila* by using germline transformation. All that is required for integration of a P-element into the genome of *Drosophila* is the 31bp inverted terminal repeats which lie at the extreme ends of the P-element and a supply of transposase protein. Thus, sequences encoding STK61 protein can be introduced into the germline of embryos under the control of a heat-shockable promoter, as shown in figure 6.1. A 'helper' element is co-injected which provides transposase, but cannot itself integrate into the genome due to mutated terminal repeats.

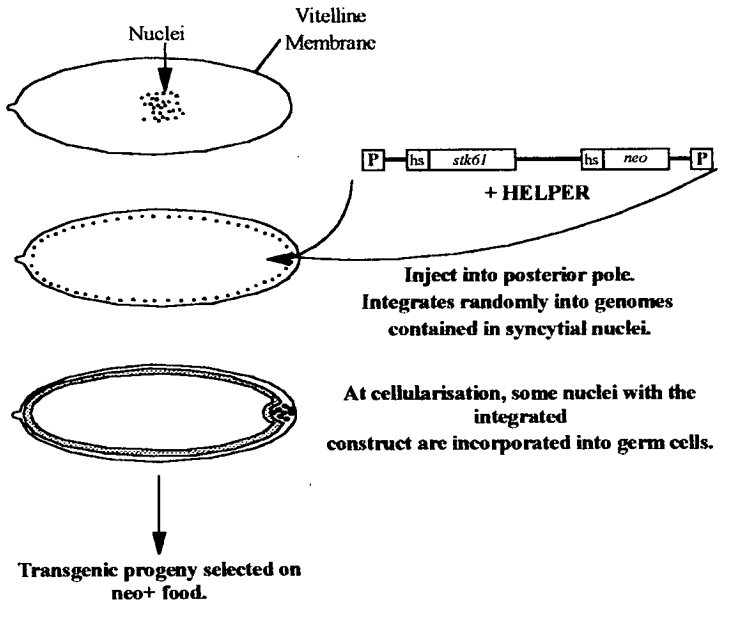


Figure 6.1
Germline transformation in *Drosophila*. hs=*hsp70* heat-shockable promoter, P=P-element terminal repeats, *neo*=neomycin resistance gene. See text for details.

This experiment is currently being carried out by Diane Harbison, in our lab. It is difficult to predict exactly what phenotypes ectopic expression of *stk61* would produce. However, based upon the predictions discussed above, we might expect to see a number of sex-specific phenotypes such as gametogenic, muscular, cuticular and behavioural defects. The results of this experiment will allow predictions to be made about what phenotypes may be produce by 'site-selected' P-element mutagenesis (see above).

The ability to ectopically express *stk61* would also enable identification of genes which genetically interact with *stk61*, as shown in figure 6.2.

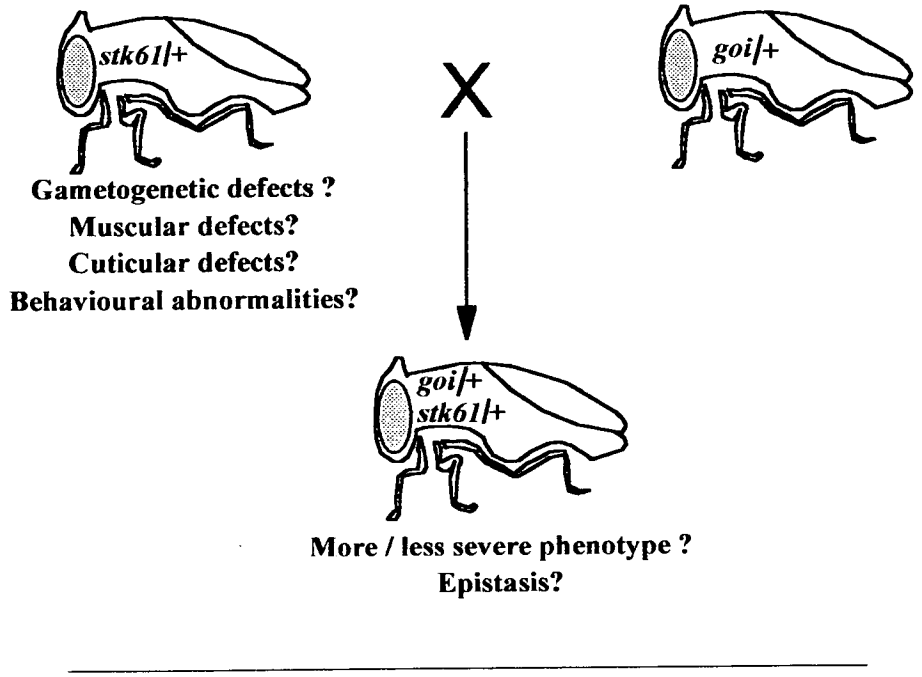


Figure 6.2
Use of ectopic expression of *stk61* to identify genetic interactions. goi=gene of interest. See text for details.

Clearly, choice of the 'gene of interest' shown in figure 6.2 will depend upon evidence such as the localisation of STK61 protein and the phenotypes caused be *stk61* miss-expression, which will help to identify particular genetic pathways in

which *stk61* might play a part. Since *stk61* encodes a S/T kinase, possible genes of interest might include genes known to be involved in signal transduction pathways, such as the *ras* gene which is involved in a number of different transduction processes in the developing embryo. We might expect ectopic *stk61* expression to cause a modulation of *ras* phenotypes. Such *ras*-mediated processes might include specification of the terminal regions of the embryo, or specification of the R7 photoreceptor.

In the longer term, the transcriptional regulation of the *stk61* gene could also be investigated by germline transformation. Constructs containing LacZ reporter gene sequences under control of genomic DNA sequences, 5' to the transcriptional start site of cloned cDNA's, may help to determine whether transcriptional regulation plays any part in the production of sex-specific *stk61* transcripts. It is possible that separate promoter elements are responsible for production of the various *stk61* transcripts, which influences the splicing pattern of these transcripts. This type of regulation is seen in the *Sxl* gene, where transcription from the early promoter, P_E, results in the production of active fully-spliced *Sxl* transcript, despite the lack of active Sxl protein (See chapter 1 for details). Transcripts produced from the late promoter, P_L, however, require the action of Sxl protein for active fully-spliced *Sxl* transcript to be produced. We have seen that intron 4 is spliced out of the testis-specific *stk61* cDNA, even in pseudo-testes, which lack active Tra-2 and Tra (figure 4.3). Yet, the removal of this intron appears to require Tra and Tra-2. It may be that a system analogous to that of *Sxl* exists, whereby transcription of *stk61* from a testis-specific promoter somehow bypasses the need for splicing regulation. When specific *stk61* promoter sequences have been delimited, factors which bind to these elements could be identified by gel-shift assays and protein expression library screening.

Germline transformation could also be used to construct flies expressing LacZ reporter gene transcript containing the *stk61* TCE elements in 5' UTR sequences.

This would enable a convenient assay to test the translational control of these TCE elements, and the effect of mutation of the elements.

In addition, the function of the cDNA11-specific 3'UTR could be investigated by germline transformation. Expression of a LacZ-encoding transcript, fused to the cDNA11-specific 3'UTR, would reveal whether the 3'UTR acts to mediate translational control or specific mRNA localisation.

Clearly, the *stk61* locus is a complex one, which will require a great deal of further study to fully understand its regulation and function. The work presented here identifies the *stk61* gene as a good candidate for a sex differentiation gene which has a number of features which are unique and will make this gene highly interesting as a possible inroad into the largely unknown territory of somatic sex differentiation in *Drosophila*.

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APPENDIX

PUBLICATIONS

DEVELOPMENTAL BIOLOGY 172, 353–376 (1995)

REVIEW

The Developmental Consequences of Alternate Splicing in Sex Determination and Differentiation in *Drosophila*

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INTRODUCTION

Many eukaryotic genes generate alternately spliced transcripts which can produce different proteins or which have altered translational controls. One of the most direct demonstrations that alternately spliced forms of transcripts lead to different developmental consequences lies within the sex determination pathway of *Drosophila*. The *doublesex* (*dsx*) gene at the end of this pathway in somatic cells encodes two differently spliced transcripts, one specific for males and one specific for females (Burtis and Baker, 1989). These encode proteins with a common DNA binding region and a sexually unique carboxy terminus; DSX^M and DSX^F act as transcription factors and have opposing activities. The main developmental consequences are the repression of a set of downstream female-specific differentiation genes by DSX^M in males and the repression of certain male characteristics by DSX^F in females (for reviews see Slee and Bownes, 1990; Steinmann-Zwicky *et al.*, 1990; Ryner and Swain, 1995).

Many of the sexual differences between male and female *Drosophila* are controlled by the two alternate products of the *dsx* gene (Burtis and Baker, 1989). They direct both the determination of sex-specific characteristics in the imaginal cells and the maintenance of determination throughout subsequent cell divisions. This regulation leads to the final differentiation of male or female genitalia, the differences in pigmentation patterns in the abdomen of each sex and in other sex-specific bristle patterns, such as the sex comb on the first leg of the male. Differences between the sexes in the pattern of nerve cell divisions are also directed by the two related DSX proteins (Taylor and Truman, 1992), as is the selection between the male or female development of the gonad into a testis or ovary and its subsequent differentiation (Szabad and Nöthiger, 1992). The *yolk protein* genes which are expressed in the adult female fat body are targets of the DSX protein (Burtis *et al.*, 1991) and their yolk protein products are essential for oocyte development.

However, the use of alternate splicing in sexual development in *Drosophila* is not limited to *dsx*. Alternate splicing of *dsx* transcripts is controlled by the products of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes (Nagoshi *et al.*, 1988). *tra* RNA is also alternately spliced, in this case with the dramatic consequence that in males no functional protein product is made (Butler *et al.*, 1986; McKeown *et al.*, 1987), whereas in females an RNA binding protein is produced which interacts with the *tra-2*-encoded RNA binding protein (Belote and Baker, 1982), directing the female-specific splicing of *dsx*. It seems likely that *tra* and *tra-2* have other targets in addition to *dsx* that are important for sexual development, since several aspects of sexual dimorphism depend upon the *tra/tra-2* genes but are independent of *dsx* (Taylor *et al.*, 1994). This includes courtship behaviour; the development of abdominal cells, which produce a female pheromone; and the correct innervation of nerves needed for the development of a male-specific muscle (Lawrence and Johnston, 1986; Taylor, 1992).

The female-specific splicing of *tra* RNA is itself directed by an alternately spliced gene product. This is encoded by the *Sex-lethal* (*Sxl*) gene that is at the head of the sex-determination hierarchy (Cline, 1984, 1993). Sxl produces many transcripts, including several specific to female somatic cells, that generate a functional RNA binding protein. The male mRNA from *Sxl* does not encode a functional protein (Bell *et al.*, 1988). The SXL protein is known to direct the female-specific splicing of its own RNAs as well as that of *tra* (Inoue *et al.*, 1990; Bell *et al.*, 1991; Horabin and Schedl, 1993a,b; Samuels *et al.*, 1994).

The female product of *Sxl* also directs suppression of the

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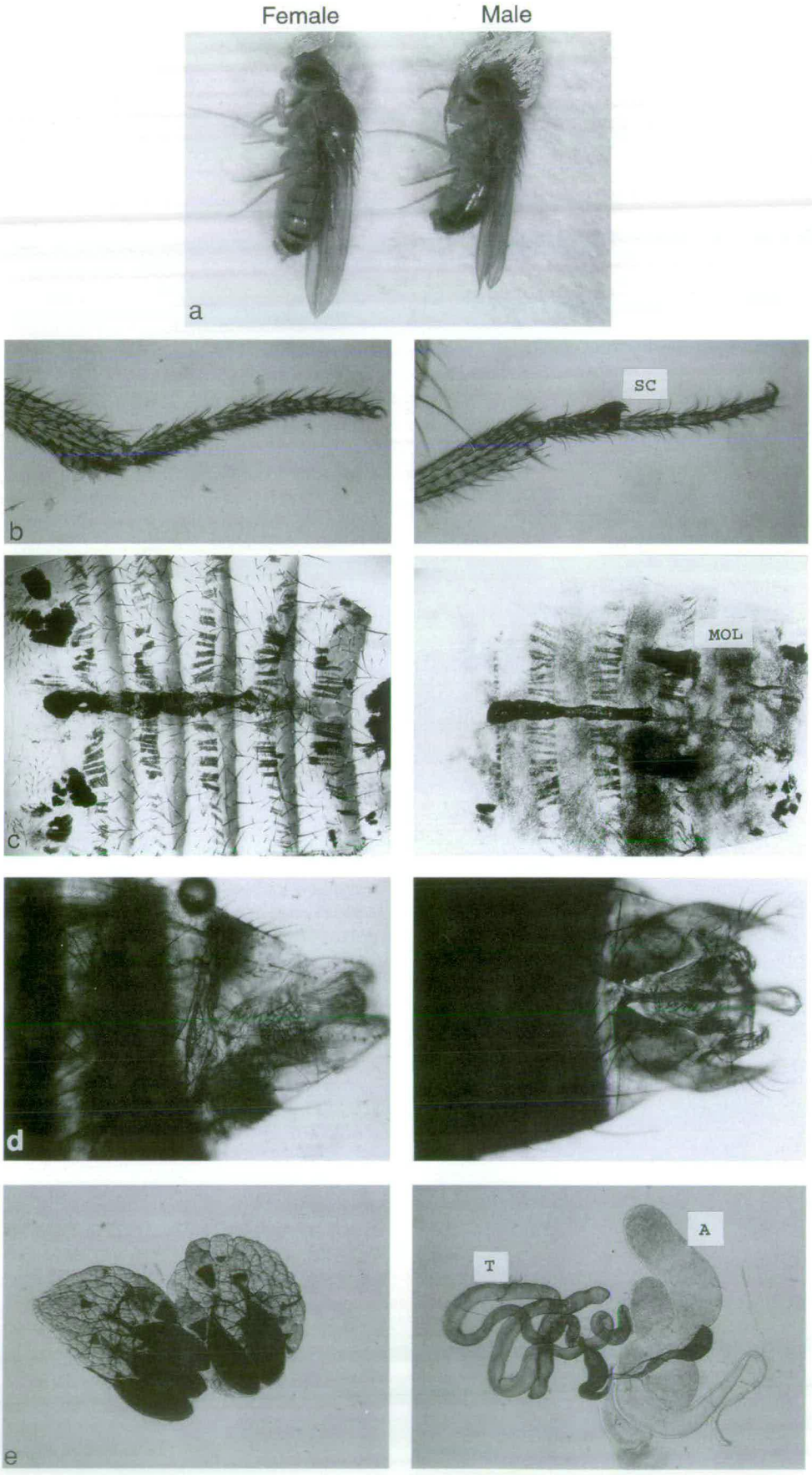
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hyperactivation of the X-chromosomes in females (Baker and Belote, 1983; Lucchesi and Manning, 1987; Baker *et al.*, 1994). X-chromosome hyperactivation is a process that is essential for dosage compensation in males, as they carry only one X-chromosome. It is this function of *Sxl* which leads to sex-specific lethality when *Sxl* is inappropriately activated or repressed. Female differentiation of the germline also depends upon *Sxl* (Steinmann-Zwicky, 1992). From its multiple functions, some of which are not mediated through the activities of *tra* and *tra-2*, it seems that *Sxl*, like *tra*, must have more target genes that remain to be discovered, namely those involved in dosage compensation, size (females are larger than males), and germline differentiation. Thus this pathway provides excellent insight into how alternate splicing can be used to ensure that very different developmental decisions are taken during development. Its branched nature, with multiple targets at each point in the hierarchy, shows how a complex network of interrelated processes can be controlled at the level of mRNA processing. Figure 1 shows the morphological sexual differences between adult males and females, and Fig. 2 summarises the sex determination pathway in somatic cells.

SOMATIC SEX DETERMINATION

The primary determinant of sex in flies is the number of X-chromosomes to sets of autosomes, the X:A ratio (see reviews by Slee and Bownes, 1990; Steinmann-Zwicky *et al.*, 1990; Cline, 1993; Ryner and Swain, 1995). Those flies that have one X-chromosome to two sets of autosomes (X:A = 0.5) are male, whilst those that have an X:A ratio of 1 (i.e., two X-chromosomes to two sets of autosomes) are female. The process of somatic sex determination is largely cell autonomous; there appears to be no hormonal component. This can be inferred from studies on gynandromorph flies possessing both XO and XX cells which develop into male and female tissue autonomously. Since the male and female cells are exposed to the same compounds circulating in the haemolymph, a hormonal influence can be excluded. The Y-chromosome does not play a role in sex determination, unlike the process in mammals; rather it carries genes which are required to complete spermiogenesis (Fuller, 1993).

Activation of Sex-lethal

The X:A ratio is assessed by a number of zygotic loci that are located on the X-chromosome. These activate *Sex-lethal* and are known as numerator elements (*sisterless-a* (*sis-a*), *sisterless-b* (*sis-b*), and *runt*). There are also repressor elements (or denominators) on the autosomes (*deadpan* (*dpn*)) (for review see Parkhurst and Meneely, 1994). An additional X-linked locus known as *sisterless-c* (*sis-c*) has also been identified; this appears to act as a numerator element although its effects are weaker than those of either *sis-a* or *sis-b* (Cline, 1993). Numerator elements act as feminising elements, while the known denominator acts as a masculinising element (Cline, 1993). Alterations in the dosage of these elements leads to sex-specific lethality. A reduction in the numerator dose results in female-specific lethality, while an increase is lethal to males (Cline, 1988; Younger-Shepherd *et al.*, 1992). The denominator element exhibits the reciprocal phenotype, an increase in dosage resulting in female lethality. The assessment of the X:A ratio affects the activation of the gene *Sxl*. Cells that have an X:A ratio of 1 (i.e., female) activate *Sxl*, while those that have an X:A ratio of 0.5 (i.e., male) do not activate *Sxl*.

Several of the numerator elements and the denominator element *deadpan* have been cloned and characterised at the molecular level. *sis-a* is a member of a family of transcription factors known as basic leucine zippers (Erickson and Cline, 1993a). *sis-b* (which corresponds to *scute* α/T_4, a member of the *achaete/scute* complex) encodes a basic helix–loop–helix protein (bHLH) (Cline, 1988; Torres and Sánchez, 1989; Erickson and Cline, 1993b). The pair-rule segmentation gene *runt* has also been implicated in sex determination (Duffy and Gergen, 1991). The predicted *runt* protein shows homology with a family of transcriptional regulators, including the polyoma enhancer binding protein. Interestingly, this gene, unlike *sis-a* and *sis-b* which act throughout the embryo, appears to affect sex determination only in the central region of the trunk. To date, only one denominator element has been identified, the proneural gene *dpn*, which encodes a bHLH protein. As expected for a denominator element, alterations in the gene dosage of this locus result in sex-specific lethality (Younger-Shepherd *et al.*, 1992; Cline, 1993).

In common with many other processes in *Drosophila*, sex determination relies upon maternally contributed RNAs laid down in the oocyte during oogenesis. One of these is encoded by the gene *daughterless* (*da*) (Caudy *et al.*, 1988; Cronmiller *et al.*, 1988). As its name suggests, females mutant at one allele of this locus (da^1) do not produce any female progeny. Again, *da* encodes a protein that contains a bHLH domain. The neural locus *extramacrochaetae* (*emc*) has been predicted to act as a negative regulator of *Sex-lethal*. The protein contains a HLH domain but does not contain the basic residues which are essential for DNA binding (Younger-Shepherd *et al.*, 1992; Bier *et al.*, 1992).

FIG. 1. Morphological differences between males and females. (a) Male and female flies; note the male is smaller than the female. (b) Male and female foreleg; note the sex comb on the male. (c) Male muscle and equivalent segment in female (photographs courtesy of Dr Peter Lawrence, MRC, Cambridge). (d) Male and female genitalia and abdominal pigmentation; note the more extensive pigmentation in the male. (e) Male and female gonad. A, accessory gland; MOL, muscle of Lawrence; SC, sex comb; T, testis.

In this case, maternally contributed *emc* may act by binding with the other HLH-containing proteins to form nonfunctional heterodimers. Recently, another protein which interacts with hairy-related bHLH proteins has been identified (Paroush *et al.*, 1994). The protein encoded by *groucho*, has been shown to interact with *dpn* and may act as a transcriptional corepressor in conjunction with other bHLH proteins. The *hermaphrodite* (*her*) locus has multiple roles during sex determination. Maternally contributed HER appears to act as a positive regulator of *Sxl* activation and also affects the process of dosage compensation. The zygotic function of *her* is not rescued by the constitutive expression of either *Sxl* or *tra*. *dsx* splicing is unaffected in intersexual flies resulting from *her* zygotic mutants. This implies that the zygotic function of *her* may be similar to that of *intersex* (*ix*), acting in parallel with or downstream of *doublesex* (Pultz *et al.*, 1994; Pultz and Baker, 1995). *her* has recently been characterised at the molecular level. It encodes a zinc finger protein which may function as a transcription factor (Ryner and Swain, 1995). These proteins activate *Sex-lethal* at the level of transcription (Keyes *et al.*, 1992). The various bHLH proteins are able to interact to produce homo- or heterodimers which then bind to DNA, activating transcription. The ability of these proteins to form heterodimers resulting in *Sxl* activation was demonstrated by the inappropriate expression of another bHLH protein, the pair-rule gene *hairy* (Parkhurst *et al.*, 1990). When *hairy* protein is expressed ectopically under the control of the *hunchback* promoter, it is a female lethal. It was shown that HAIRY interacts with the other HLH proteins to form heterodimers which could not bind to DNA, preventing *Sxl* from being activated in females. The gene *sans fille* (*snf*) (known also as *liz* (Steinmann-Zwicky, 1988) and *fs(1)1621*) represents a maternal effect gene that is required for activation of *Sxl* in both the germline and the soma. *snf* has been characterised at the molecular level and shows significant sequence and functional homology with the U1A snRNP protein (Flickinger and Salz, 1994). The *virilizer* locus has also been implicated in the process of sex determination (Hilfiker and Nöthiger, 1991). However, it is not yet clear where in the hierarchy *vir* acts. It appears to function upstream of *tra* to modulate *Sxl* activity but whether it acts directly on *tra*, *Sxl*, or both has not been established.

It is interesting to note that several of the genes involved in the assessment of the X:A ratio also have a function later in development, during neurogenesis. The maternal product of *daughterless*, for example, is required during sex determination; the zygotic function is required later for development of the peripheral nervous system (Caudy *et al.*, 1988). *sis-b* (a member of the *achaete/scute* complex), *deadpan* (proneural gene), and *extramacrochaetae* (neural gene) are also involved in both of these processes. These bHLH proteins may interact to constitute a genetic switch, such that the ratio of positive (numerators) to negative (denominators) regulators determines a cell's fate by affecting the transcriptional activity of downstream genes.

Sex-lethal

Sex-lethal plays a pivotal role in the processes of both somatic and germline sex determination as well as in the process of dosage compensation (Fig. 2) (Baker, 1989; Cline, 1993).

The structure of *Sex-lethal* is complex, with 10 exons and two promoter regions dispersed over a region of 25 kb (Samuels *et al.*, 1991) (Fig. 3). The use of the different promoters, different exons, and different polyadenylation sites leads to the production of at least 10 different RNA species, with varying patterns of expression. Three transcripts are specific to the male (4.3, 3.3, and 2.1 kb) and four to the female (4.1, two transcripts of 3.1, and 1.9 kb). One of the 3.1 kb transcripts and the 1.9-kb transcript are probably germline-dependent, since their levels of expression are reduced in abdomens that contain no ovaries. In addition, the initial activation of *Sex-lethal* results in the production of early transcripts in the female embryo (Fig. 3). These transcripts are derived from the early promoter (P_E) in response to the X:A signal (X:A = 1). Alternate splicing and the use of different polyadenylation signals give rise to three transcripts of 3.7, 2.6, and 1.6 kb. *In situ* hybridisation to whole-mount embryos indicates that these early transcripts are present in the embryo prior to pole-cell formation (Keyes *et al.*, 1992) (Fig. 4). The signal peaks in embryos at about nuclear division 12 and begins to decline until germ band extension, when it can no longer be detected. Neither early transcripts nor protein are detected in the pole cells (these are the germline primordia, Fig. 4). Not surprisingly, these transcripts are not present in flies mutant for *da*. The early proteins have been suggested to act in establishing the positive feedback loop for *Sex-lethal* autoregulation as they are present in the embryo at the time when the early functions of *Sex-lethal* occur (Salz *et al.*, 1989).

The next step in the regulation of *Sex-lethal* occurs at the level of RNA splicing. The main difference between the male transcripts and those of the female is in the incorporation of exon 3 (male-specific exon) in the male. This exon contains several translational stop codons, resulting in the formation of a truncated protein in the male. In the female, SXL protein translated from the early transcripts directs the splicing pattern of the later transcripts such that the male-specific exon is spliced out, enabling a full-length protein to be produced. These proteins maintain the productive mode of splicing.

Sequence analysis of the female cDNAs indicates that they contain a long open reading frame (ORF) which extends from exon 2 to at least exon 8 (see Fig. 3). This gives a predicted protein product of approximately 354 amino acids (Bell *et al.*, 1988; Samuels *et al.*, 1991). The sequence shows two conserved domains, RMM1 and RMM2, which show significant sequence homology to a conserved RNA binding domain found in other RNA binding proteins (RNPs) (Bell *et al.*, 1988). This family of proteins is able to bind both RNA and single-stranded DNA and functions by binding to various RNA species (including its own) to direct their

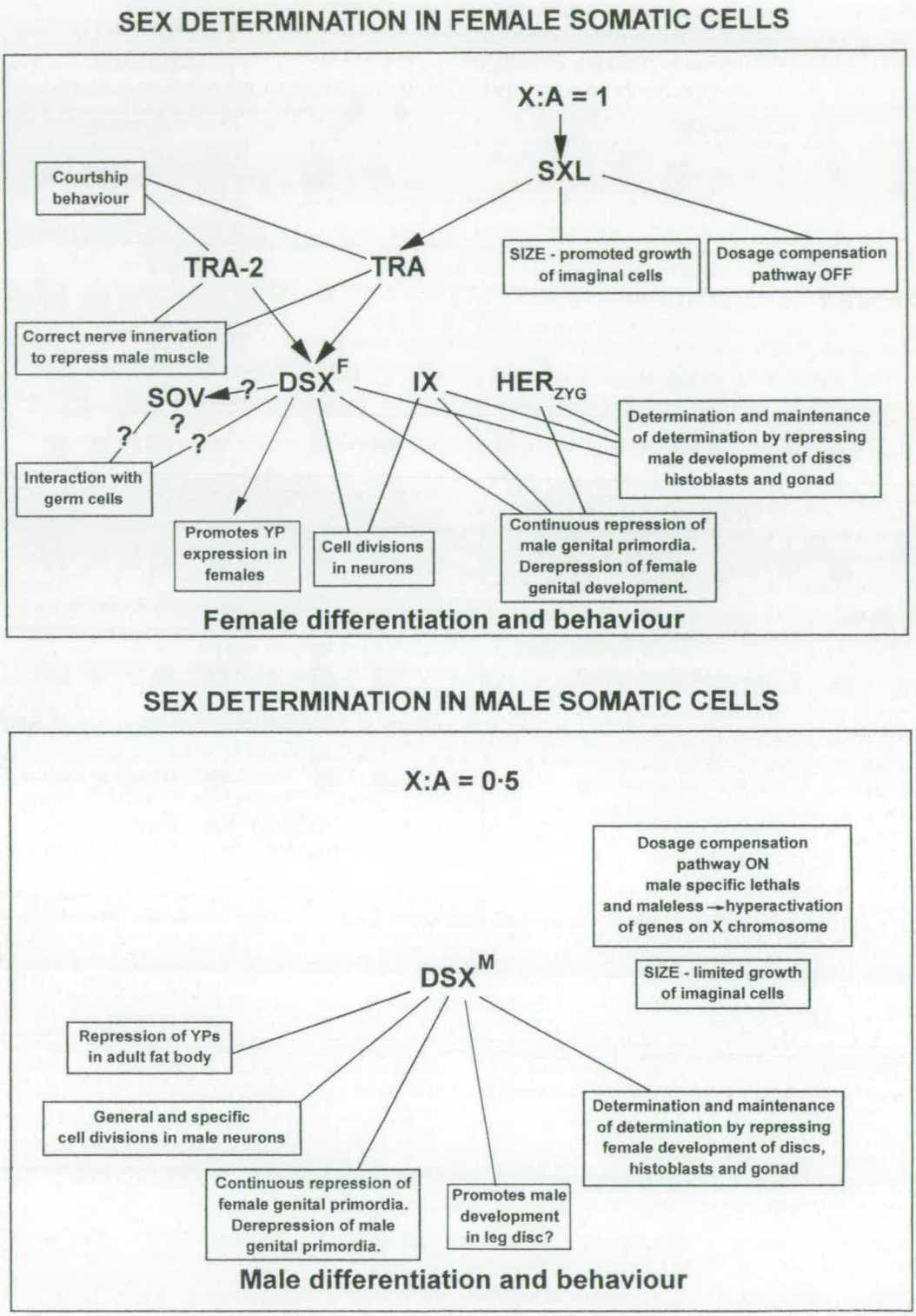


FIG. 2. Diagrams of sex determination in female and male somatic cells. X:A, X chromosome to autosome ratio; SXL, *Sex-lethal* transcript is spliced in female mode and makes a functional protein; TRA, *transformer* transcript is spliced in female mode in the presence of SXL protein and encodes a functional protein; TRA-2, *transformer-2* product is required; DSX^F, *doublesex* transcript is spliced in female mode in the presence of TRA and TRA-2 protein and makes a female-specific protein which regulates the expression of downstream genes; DSX^M, in the absence of TRA protein, *doublesex* transcript is spliced in the male mode to produce a male-specific protein that regulates the expression of downstream genes; IX, *intersex* product interacts with DSX^F to regulate downstream genes in females and affects courtship behaviour in males; HER_{ZYG}, the zygotic product of the *hermaphrodite* (*her*) gene is required for female differentiation independently of DSX. Arrows represent interactions shown experimentally. Arrows with question marks demonstrate possible interactions. *tra* does not induce germ cells but is required for differentiation of female germ cells.

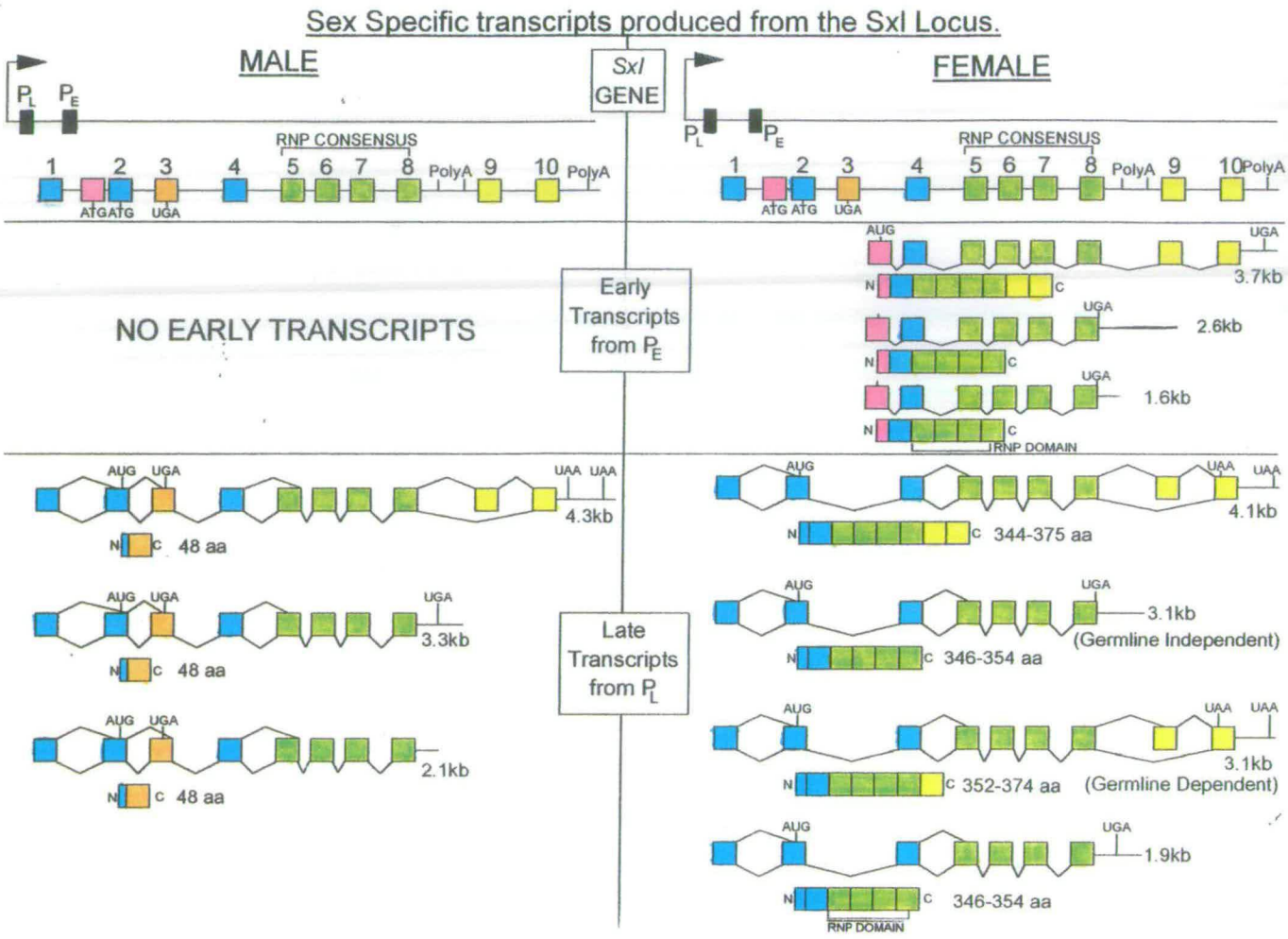
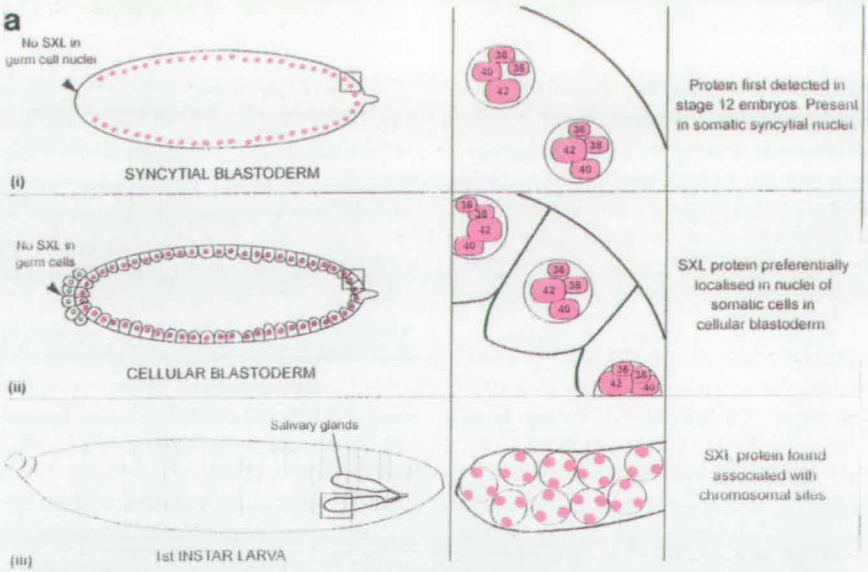


FIG. 3. The structure of the *Sex-lethal* transcription unit. The positions of the two promoters (early P_E and late P_L) are indicated. In the female, early proteins from P_E direct the splicing of the late transcripts from P_L into the female mode. Different forms of the protein are produced by the use of different splice and polyadenylation sites. These proteins all contain the ribonucleoprotein (RNP) binding domain that is required for *Sxl* function. In the male, where no early transcripts are produced, no splicing of the late transcripts occurs. Consequently the third exon (which contains stop codons) is included, producing a truncated protein that does not contain the RNP domain.

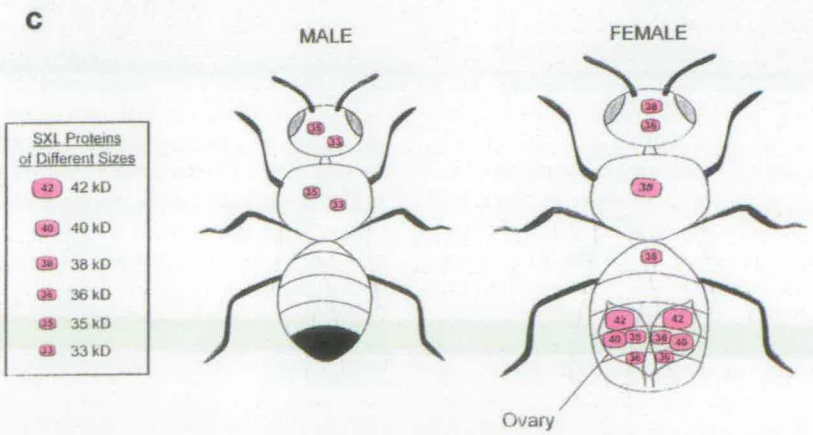
splicing pattern (Samuels *et al.*, 1994). The mechanism by which this sex-specific splicing is achieved will be discussed in the section dealing with the *tra* and *tra-2* genes.

As expected, the presence of SXL protein differs between the two sexes. It is present in female embryos and absent in male embryos (Bopp *et al.*, 1991) (see Fig. 4). In addition, the open reading frames differ among the different classes of female-specific RNAs, depending on the different splice sites and polyadenylation signals. Most of these differences occur at the carboxy terminus of the protein and all of the

FIG. 4. Developmental distribution of SXL protein. (a) In the embryo (i) SXL is found in all the syncytial nuclei except those which form the pole cells. At cellular blastoderm (ii), SXL remains localised to the nuclei. During the later stages of development, SXL can be detected in probably all somatic tissue such as the third instar larval salivary gland nuclei (iii). (b) Embryos stained using anti-SXL antibody. Antibody is stained pink and so female embryos appear pink and male embryos remain blue. No SXL protein can be detected in the pole cells (white arrow). (c) SXL protein can also be detected in male and female adults. In the female, two major species of protein (36 and 38 kDa) can be detected in all tissues. Additionally, two minor species of 40 and 42 kDa can be detected in the ovary. In the male, two smaller species of 33 and 35 kDa can be detected in the head and thorax. The functional significance of these protein varients is not known.


a
No SXL in germ cell nuclei
(i)
SYNCYTIAL BLASTODERM
Protein first detected in stage 12 embryos. Present in somatic syncytial nuclei
No SXL in germ cells
(ii)
CELLULAR BLASTODERM
SXL protein preferentially localised in nuclei of somatic cells in cellular blastoderm
Salivary glands
(iii)
1st INSTAR LARVA
SXL protein found associated with chromosomal sites


b


c
MALE
FEMALE
SXL Proteins of Different Sizes
42 kD
40 kD
38 kD
36 kD
35 kD
33 kD
Ovary

predicted female products contain both RNP domains. The distribution of the SXL protein was assessed using monoclonal antibodies raised against sequences present downstream of the male-specific exon. Two prominent proteins (38 and 36 kDa) were detected in the adult female but not in the male. These two variants derive from alternate utilisation of the 3′ splice site of exon 5. There was a differential distribution of these proteins within the fly. Both these proteins could be detected in the ovaries and head. In dissected carcasses containing no ovaries, the 38-kDa protein was the major species, with lower levels of the 36-kDa protein present. The predominant species in the thorax was the 38-kDa protein. In addition to these two species, two other minor forms of the protein (40 and 42 kDa) could be detected in ovaries and early embryos (Bopp *et al.*, 1991).

Surprisingly, SXL protein could also be detected in the adult male. Two species of 35 and 33 kDa are detected in the thorax and head; these are more prominent in the head than in the thorax and cannot be detected in the abdomen. Protein levels are 20–40 times lower than those detected in females. These proteins have been demonstrated to be derived from the *Sxl* locus, since they are absent in males carrying deletions for *Sxl*. They are similar to the low-abundance SXL proteins, which are also detected in females. It is possible that these proteins are produced from translational initiation codons downstream of exon 3. However, they do not appear to function in splicing (Bopp *et al.*, 1991), since no female-like *Sxl* or *tra* transcripts are detected in males.

SXL protein has been shown to be preferentially localised to the nucleus. In the interphase nucleus during embryogenesis, SXL protein can be detected as regions of intense staining superimposed on more diffusely staining nucleoplasm, suggesting that high levels of SXL protein have been accumulated (Bopp *et al.*, 1991).

Sex-lethal and Germline Sex Determination

In contrast to the process of sex determination in the soma, the mechanism by which sex determination occurs in the germline is still unclear. The known factors are shown in Fig. 5. As described previously, the *Sex-lethal* early transcripts (and protein) are not present in the germline primordia (Bopp *et al.*, 1991; Keyes *et al.*, 1992). In addition, the numerator elements *sis-a*, *sis-b*, and *runt* have been shown by pole-cell transplantation experiments not to be essential for *Sxl* activation in the germline. Pole cells from embryos mutant for all three of these loci have been shown to produce functional germ cells when transplanted to a wild-type background (Granadino *et al.*, 1993; Steinmann-Zwicky, 1994). In contrast to the somatic process, the genes *tra*, *tra-2*, and *dsx* have no function in female germline development (Marsh and Wieschaus, 1978; Schüpbach, 1982). *tra-2*, however, is required in males for the production of motile sperm (Fuller, 1993). Germ cells that are 1X:2A (i.e., male) attempt to develop as spermatocytes irrespective of the sex of the surrounding somatic tissue. In a female somatic background, these germ cells assume an intersexual identity. *Sxl* activation in the germline, in common with the process in the soma, is cell autonomous and is essential for female development. However, 2X:2A germ cells (i.e., female) also require an inductive signal to start and/or complete sexual development, which depends upon the phenotypic sex of the surrounding soma. Consequently, intersexual cells are produced in a male somatic background while oocytes develop in a female background. Any spermatocytes which are produced arrest in the primary spermatocyte stage. This suggests (at least in the female) that inductive interactions are required between the soma and the germline in order to direct the correct sex determination of the germ cells (Nöthiger *et al.*, 1989; Steinmann-Zwicky, 1992). This signal is established during embryogenesis after somatic sex determination has occurred and therefore the correct expression of the downstream sex-determining genes is required. The inductive interactions may be mediated through the action of *tra-2*. XX flies mutant for both *dsx* and *tra* express significant levels of female *Sxl* activity. In *tra-2* mutant XX individuals, *Sxl* is expressed in the male mode. Since *tra-2* is not expressed sex-specifically, it has been postulated that it interacts with an unknown gene (which may be under the control of *Sxl*) to control the inductive signal from the soma to the germline (D. Bopp, personal communication). This inductive signal has been postulated to act in one of two ways: either 2X germ cells undergo male development unless they receive an inductive signal from the female soma resulting in *Sxl* activation, thus promoting female development; or 2X germ cells undergo oogenesis unless they receive an inducing signal from the male soma to repress *Sxl*, allowing male development to occur.

Several loci have been isolated which appear to function during germline sex determination. Mutations in the genes *snf* (Salz, 1992), *fl(2)d* (Granadino *et al.*, 1990), and some alleles of the *ovarian tumour* (*otu*) locus (Pauli *et al.*, 1993) result in the formation of multicellular cysts in the ovary. This phenotype is similar to that observed with several *Sxl* germline-specific mutations. SXL protein is absent in the ovaries of flies mutant for *snf* or otu^{onc} genes (Bopp *et al.*, 1993). As described previously, the *snf* locus encodes a protein which exhibits homology to the U1A snRNP protein. It is therefore likely that the SNF protein acts to establish the correct splicing of *Sxl* transcripts in both the soma and the germline (Bopp *et al.*, 1993; Oliver *et al.*, 1993; Flickinger and Salz, 1994). Although *otu* has been cloned and sequenced, no significant homology to other sequences has been obtained. It has been postulated that these genes may act in establishing *Sxl* autoregulation. Mutations in the gene *bag-of-marbles* (McKearin and Spradling, 1990) result in sterility in both males and females, with production of undifferentiated cysts observed in both the testis and the ovary. The germ cells in these cysts are morphologically similar to germline stem cells, gonial cells or, in some cases, spermatocytes. Ovaries from these flies have normal levels of SXL but the localisation of the protein is perturbed (Bopp *et al.*, 1993). Unlike the previous classes of mutants, mutations at the *ovo* locus are still able to produce rudimentary

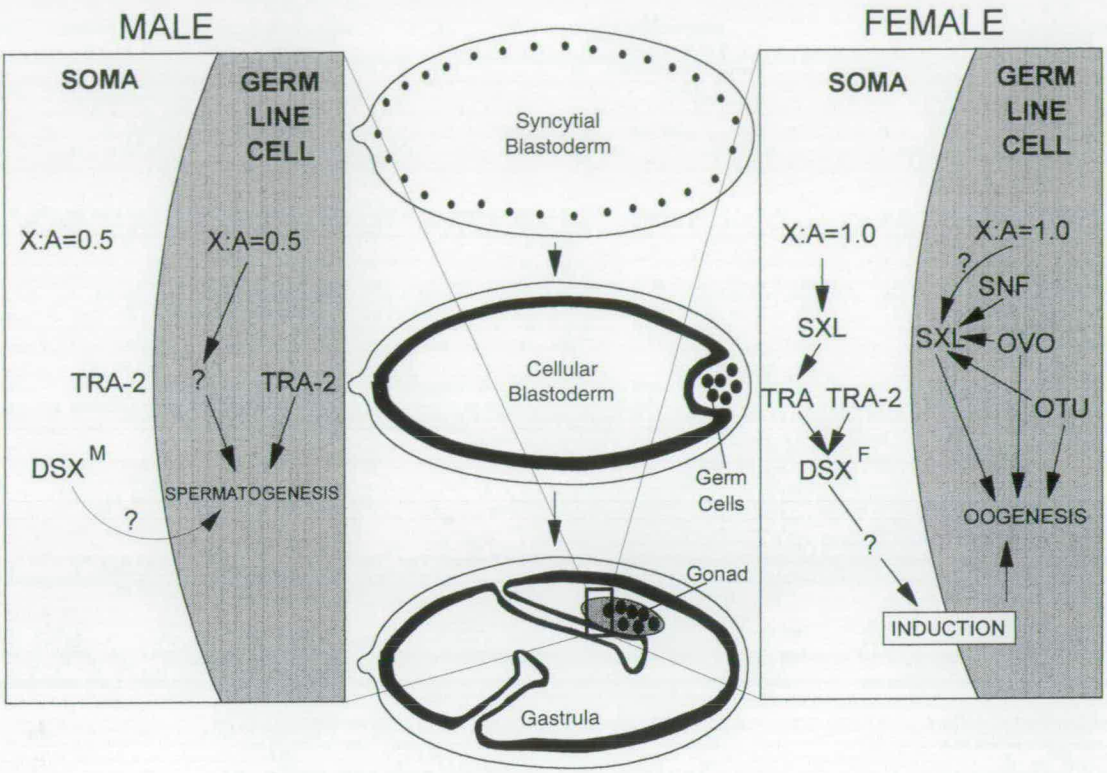


FIG. 5. A schematic representation of the process of germline sex determination. In the female, the process of germline sex determination differs from the process in the soma. Inductive interactions with the soma (mediated by the genes of the somatic sex determination hierarchy) are required, as well as a cell autonomous signal mediated by SXL. The signal from the X:A ratio activates SXL. This, in conjunction with the OVO and *ovarian tumour* (OTU) protein and the inductive interactions from the soma, determines the sex of the germline. In the male, there does not appear to be a repressive inductive interaction with the soma. Germline sex determination appears to be partly cell autonomous. DSX^F, *doublesex* female-specific protein; DSX^M, *doublesex* male-specific protein; OTU, *ovarian tumour* protein; OVO, *ovo* protein; SNF, *Sans fille* protein.; TRA, *transformer* protein; TRA-2, *transformer-2* protein.

germ cells. For many alleles, the process of oogenesis is defective: any egg chambers that do form degenerate and do not produce functional eggs. The *ovo* gene has been characterised at the molecular level and is found to encode a zinc finger-containing transcription factor (Oliver *et al.*, 1993). The pleiotrophic gene *fused* (*fu*) also affects germline sex determination (Bopp *et al.*, 1993; Oliver *et al.*, 1993). *fu* encodes a serine/threonine kinase and may act in soma/germline communication. *Sxl* is expressed in flies mutant for *fu* but is not correctly distributed.

Sex-lethal and Dosage Compensation

In some organisms in which one sex is heterogametic, the process of dosage compensation is essential to compensate for the functional aneuploidy that exists in the heterogametic sex. This dosage compensation can be achieved in several ways. One of the X-chromosomes may be inactivated; the 2X-chromosomes may be transcribed at a lower rate than the single X-chromosome; or the single X-chromosome may be transcribed at a faster rate than the 2X-chromosomes. In *Drosophila*, in which the male is the heterogametic sex, dosage compensation is achieved by hypertranscription of the single male X-chromosome (Mukherjee and Beermann, 1965). The incorporation of uridine into transcripts derived from the male X-chromosome relative to the autosomes was shown to be substantially higher than that of the female X-chromosome. This ensures the equalisation of levels of gene products in the male and female.

Sex-lethal and Male-Specific Lethals

Trans-acting regulators of dosage compensation have been identified. These comprise a group of four autosomal loci known collectively as the *male-specific lethals*. These are the *male-specific lethal-1* (*msl-1*), *male-specific lethal-2* (*msl-2*) (Belote and Lucchesi, 1980a), *male-specific lethal-3* (*msl-3*) (Lucchesi *et al.*, 1982), and *maleless* (*mle*) loci (Belote and Lucchesi, 1980b). As their name suggests, mutations in these genes are lethal to males but not females. It

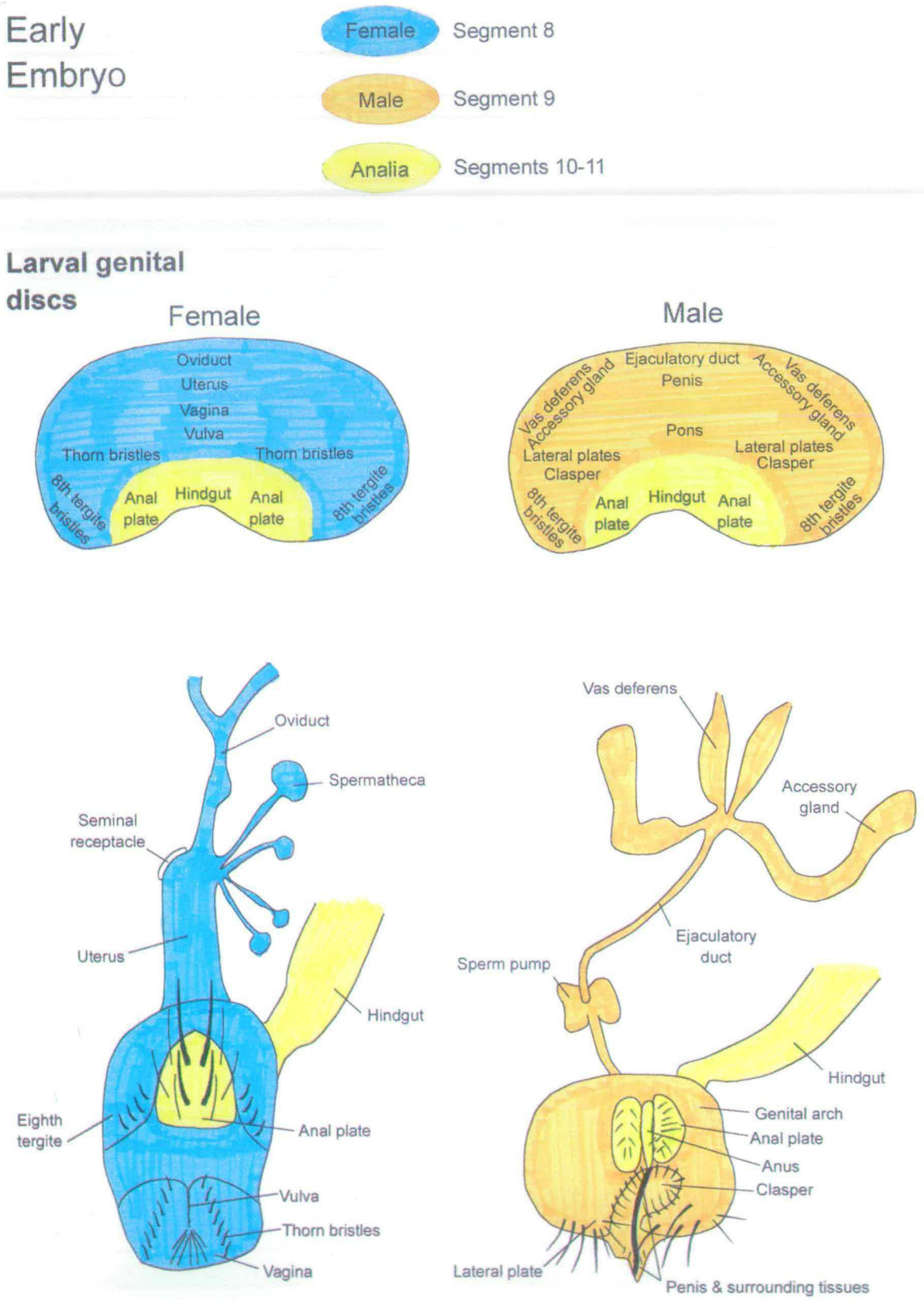


FIG. 8. The segmental origins of the imaginal disc primordia are shown. These three groups of primordial cells fuse to form the discs of the larvae and differentiate into the male and female genitalia and analia. Blue shows the derivatives of the female primordial cells, orange the male primordial cells, and yellow the analia which differentiate into different structures in male and female flies. The female primordial cells do not grow or differentiate in males and the male primordial cells do not grow or differentiate in females. They do, however, remain an integral but small population of cells within the genital disc.

EMBRYO	Female gonad DSXF - expression unknown Determination of female gonad	Fat body - both sexes DSX - expression unknown Determination of primordia for adult fat body		Male gonad DSXM - expression unknown Determination of male gonad (more cells than female)
LARVA	DSXF expressed Development of female gonad			DSXM expressed Development of male gonad
PUPA	DSXF expressed Differentiation of ovary	Differentiation of female fat body	Differentiation of male fat body	DSXM expressed Differentiation of testis
ADULT	 YPs expressed in follicle cells Trans acting factors unknown DSXF independent	Female DSXF expressed YPs transcribed in fat body Requires fat body factors DSXF enhances	Male DSXM expressed YPs repressed in fat body DSXM represses	 YPs repressed Mechanism unknown Involvement DSX unknown

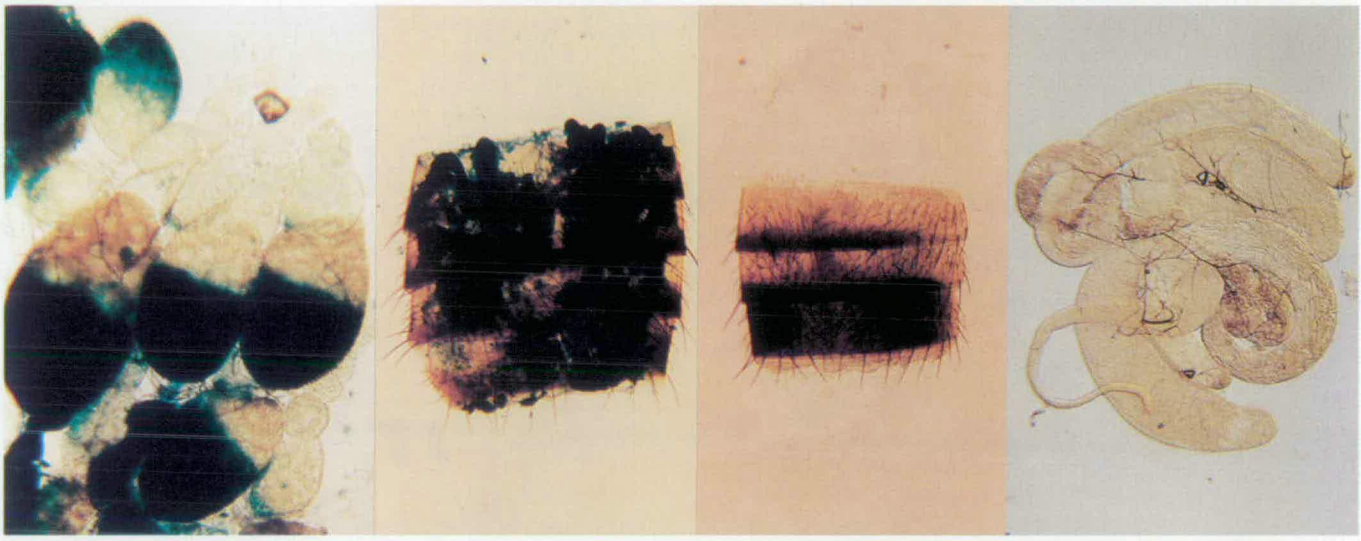


FIG. 9. The role of DSX in *yp* regulation. In the embryo DSX directs the decision as to whether the gonad will develop along a male or female developmental pathway and maintains this in the larva. In the pupa the ovary or testis differentiates and then, under the control of unknown tissue-specific factors, the *yp* genes are repressed in the testis but expressed in specific follicle cells at defined stages of oogenesis. The expression of *dsx* is unknown during fat body development. The adult fat body differentiates during metamorphosis. DSX then directs repression of the *yp* genes in the male fat body. Expression requires fat body-specific factors. Using a *lacZ* reporter the DNA sequences flanking the *yp* genes which direct this sex- and tissue-specific expression have been identified. Examples are shown for *yp3*. A fragment from −285 to +49 bp directs expression in the ovary but not the fat body. A fragment from −704 to −285 directs female fat body expression. This same construct is not expressed in the male fat body. These regions, either independently or, as shown in the figure, together, show no expression in the testis. fb, fat body; ov, ovary; hsp 70-lacZ, *heat-shock protein-70* glue promoter fused to a *lacZ* reporter gene; DSXF, *doublesex* female protein; DSXM, *doublesex* male protein.

has been postulated that these genes function in a common pathway, since mutations in one of these loci is just as detrimental as mutations in more than one. Mutations in *mle*, *msl-1*, and *msl-2* result in a 50–60% reduction in the levels of gene transcription of the X-chromosome evident in wild-type males (Baker *et al.*, 1994).

The *mle*, *msl-1*, and *msl-3* genes have been characterised at the molecular level. Proteins from these genes are expressed in both males and females; however, their pattern of distribution differs greatly between the sexes (Baker *et al.*, 1994). They are seen to associate with many sites on the male X-chromosome, but are not present on the female X-chromosome. This association leads to a significant increase in the presence of acetylated histone H_4. This observation suggests that these proteins act directly to regulate the process of dosage compensation and, in addition, that all these proteins may regulate the same set of genes. *mle*, in contrast to the rest of the *msl* proteins, is able to associate with other chromosomal locations without the interactions of the other loci. It is however unclear whether this additional binding to the chromosomes is part of dosage compensation or whether it represents another function. *mle* is allelic to *nap*, a gene which affects the activity of sodium channels (Kernan *et al.*, 1991).

Regulation by Sex-lethal

The function of *Sxl* in dosage compensation was inferred by the reciprocal male and female lethal phenotypes of gain of function and loss of function mutations. The immediate target of *Sxl* during dosage compensation is *msl-2* (Zhou *et al.*, 1995). *Sxl* functions by preventing the splicing out of a female-specific leader sequence in the *msl-2* transcripts; consequently no MSL-2 protein is produced. In the male, this sequence is spliced out, resulting in the production of functional MSL-2 protein (Zhou *et al.*, 1995). In flies which are mosaic for *Sxl* expression, MSL proteins were only associated with the X-chromosome in cells which were not expressing *Sxl*. *mle* produces multiple transcripts during development (Kuroda *et al.*, 1991), *msl-1* codes for three transcripts (Palmer *et al.*, 1994), and *msl-3* has at least three transcripts (Baker *et al.*, 1994). All of these appear to be equivalent in both males and females, showing that the regulation of these transcripts by *Sxl* is not direct. The *msl-2* 4-kb transcript can also be detected in both males and females. However, it appears that this transcript is more abundant in males than in females. Also, the female transcript appears to be slightly larger than that of the male. As described previously, this is due to the presence of an untranslated leader sequence found in the female transcript but spliced out in males. The predicted MSL-2 protein contains both a RING finger motif and a metallothionein-like domain. This protein is absent from females. MSL-2 protein has also been demonstrated to bind to the X-chromosome at the same sites as MLE and MSL-1. As discussed previously, regulation of *msl-2* may be mediated by the female-specific leader sequence. The 5′ and 3′ ends of this intron contain stretches of thymine residues similar to those found in the *Sxl* consensus binding site (Zhou *et al.*, 1995).

In polytene chromosome squashes the male X-chromosome is more open and diffuse than that of the female. It has been proposed that this altered chromatin configuration is important in allowing hypertranscription to occur. Sequence analysis of the *mle* locus has shown that it exhibits similarity with both RNA helicase A and the DEAH RNA helicases (Kuroda *et al.*, 1991). It has been proposed that *mle* forms a complex with the other *msl* proteins (similar to the spliceosome complex), facilitating hypertranscription by either increasing the rate of elongation of transcripts or removing RNA from the transcription start sites.

Other mechanisms of dosage compensation have been proposed to exist. It appears that the proteins from the *Sxl* early promoter (which, as discussed earlier, directs the autosplicing of *Sxl* transcripts) may function in directing early stages of dosage compensation which are not regulated by the *msl* loci (Gergen and Wieschaus, 1986; Bernstein and Cline, 1994). Females which are homozygous for all four *msl* loci and a null allele of *Sxl* still die. If this lethality was due to inappropriate activation of the *msl* loci, then it would be expected that the additional mutations in the *msl* loci would suppress this lethality by preventing hypertranscription. This suggests that *Sxl* may act on loci other than the known *msl*s to direct dosage compensation. The X-linked gene *runt* is an example of a gene which appears to be regulated by the action of *Sxl* and not the *msl*s. Therefore loci may exist which are also regulated by *Sxl* and act on a different set of genes. It is therefore interesting to note that alleles of *Sxl* exist that affect dosage compensation later in development which are not suppressed by mutations in *mle* or *msl-1*. As described previously, it has been suggested that all four *msl* act on the same targets. Consequently it is possible that other dosage compensation loci exist which act on a different set of target genes.

Autoregulation of Sxl

A number of uridine runs have been identified in the introns both upstream and downstream of the *Sxl* male-specific exon (exon 3) and have been implicated in SXL regulation of the female-specific splicing event (Sakamoto *et al.*, 1992; Horabin and Schedl, 1993a,b; Samuels *et al.*, 1994; Wang and Bell, 1994). Sakamoto *et al.* (1992) used a cell culture transient expression system to show that deletion of several of these U-rich motifs disrupts SXL regulation. It was observed that regulation could be restored by replacing the deleted sequences with synthetic oligonucleotides. In the first real binding study of SXL, Samuels *et al.* (1994) used gel-shifts, footprinting, and UV cross-linking with purified SXL protein to demonstrate directly that SXL binds to poly-(U) runs in RNA. Both these studies suggested that SXL may bind cooperatively to adjacent poly-(U) motifs and cooperativity has been subsequently demonstrated by Wang and Bell (1994). This study showed that SXL protein binds to many sites around the male-specific exon and that,

when bound, the proteins interact cooperatively via their N-termini. An increase in cooperativity was observed when longer RNA molecules with multiple U-rich motifs were used as a binding substrate. The authors suggest that the N-terminus of SXL may interact directly with other splicing regulatory proteins. In support of this, the hnRNP A1 has an N-terminus with a similar amino acid constituency to SXL and interacts with the splicing factor SF2/ASF.

Progress has recently been made in the elucidation of the type of mechanism used by SXL to prevent the inclusion of exon 3 in the processed female transcript. Horabin and Schedl (1993a,b) have used germline transformation to introduce altered *Sxl* minigene constructs into flies. Using RT-PCR to analyse the spliced RNA products of these mutant minigenes, they were able to ask very specific questions about what sequences around the male-specific exon are actually required for SXL regulation. Their findings have further demonstrated the importance of the poly-(U) runs both upstream and downstream of exon 3. Interestingly, it was shown that deletion of the five poly-(U) runs in the downstream intron disrupted SXL regulation much more drastically than deletion of the U runs in the upstream intron. This suggests that the critical step in preventing the inclusion of exon 3 is the blockage of the downstream intron's 5′ splice site. This is supported by the observation that when the exon 3 5′ splice site is deleted, all product in both males and females is spliced in the female mode. In summary, it appears that SXL protein prevents the inclusion of exon 3 by acting at a number of U-rich motifs which lie in the introns surrounding the exon. Exactly how this blocking occurs remains to be seen but may involve either direct modulation of splicing regulators by SXL proteins or a "nonspecific" sequestering of the whole area facilitated by SXL cooperative binding.

Sxl Regulation of tra Splicing

As well as modulating the splicing of its own transcript, SXL protein also regulates the splicing of the primary transcript from the gene transformer (*tra*). Cloning and characterisation of the *tra* gene has shown that, in female flies only, the choice of a downstream splice acceptor site prevents the inclusion of a translational stop codon. This facilitates the production of the active 211-aa TRA protein (Butler *et al.*, 1986; McKeown *et al.*, 1987, 1988; Boggs *et al.*, 1987—see Fig. 6). The observation that the area around the non-sex-specific splice site of the *tra* gene contains a uridine octamer sequence originally suggested that *tra* may be directly under the control of SXL. Transformation experiments have subsequently shown that SXL is responsible for the sex-specific splicing of *tra* nascent RNA and that the splice acceptor site containing this uridine octamer is required for this regulation (Sosnowski *et al.*, 1989). This was shown by introducing various constructs containing *tra* genomic DNA (transcribed by the hsp70 promoter) into a tra^- background via germline transformation. Deletion of the non-sex-specific splice site led to a degree of *Sxl*-independent feminisation of the male, as would be expected if the function of SXL in the female is to prevent the use of this site. Deletion of the sex-specific splice site resulted in accumulation of unspliced RNA in females, a lack of female-specific RNA, and an inability of this construct to either rescue tra^- females or transform males.

SXL blocks the non-sex-specific splice site by antagonising the essential splicing factor U2AF which binds to the same U-rich sequences as the SXL protein. SXL, however, lacks the arginine-serine "RS" repeat which is present in U2AF (Zamore *et al.*, 1992; Zhang *et al.*, 1992) as well as other splicing factors such as SF2/ASF (Ge *et al.*, 1991; Krainer *et al.*, 1991), SC35 (Fu and Maniatis, 1992), SRp20, SRp75 (Zahler *et al.*, 1992), and suppressor of white apricot ($Su(W^a)$; Chou *et al.*, 1987). The 70K U1 snRNP also contains these repeats (Theissen *et al.*, 1986; Spritz *et al.*, 1987). If the RS motif is introduced into the SXL protein, it becomes constitutively active as a splicing factor, causing splicing from the same splice site which it normally blocks (Valcárcel *et al.*, 1993).

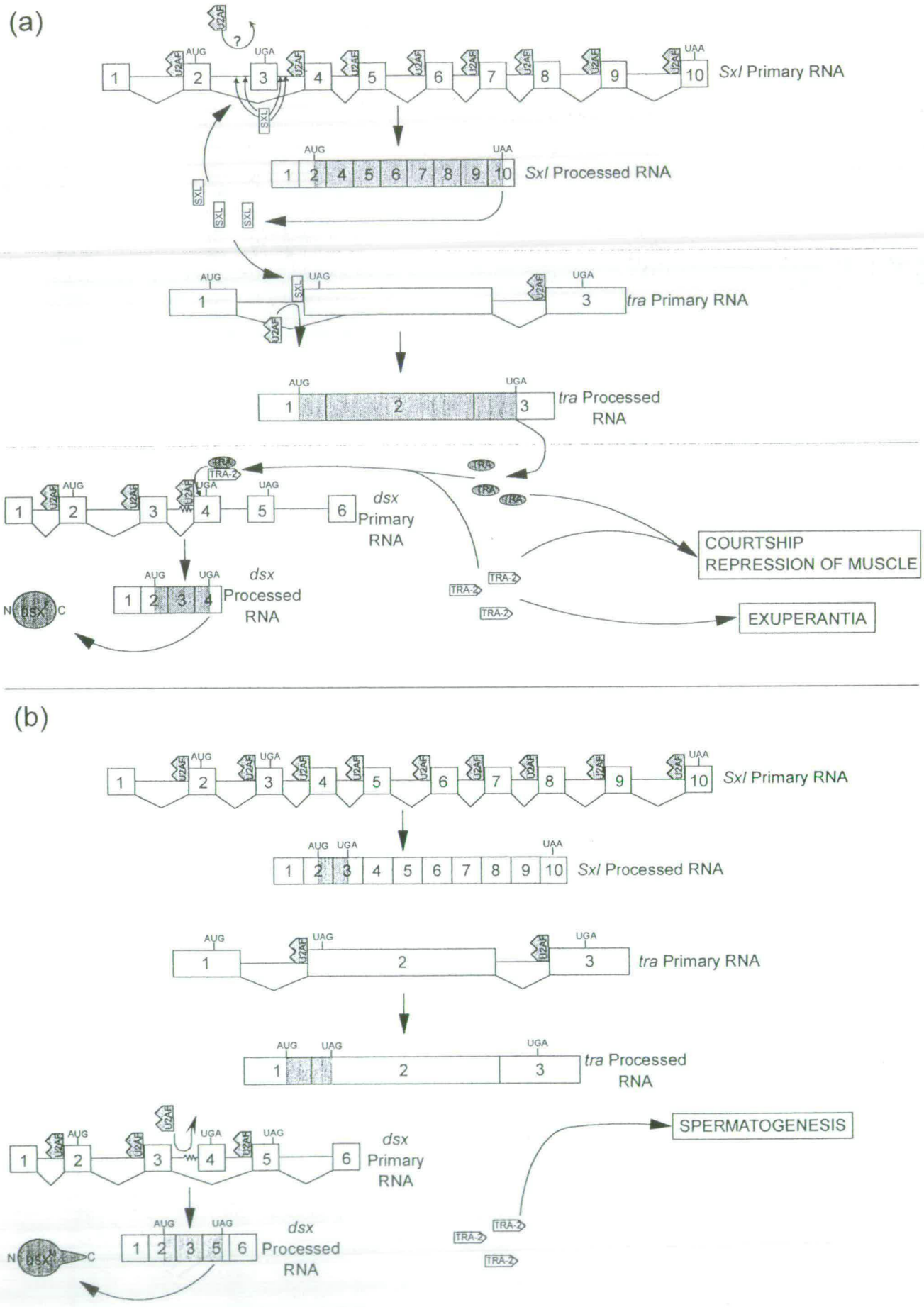
The predicted protein product from the female-specific *tra* transcript also contains an RS motif (Boggs *et al.*, 1987), indicating that the function of TRA may be to modulate splicing in the female. $Su(W^a)$ protein can be rendered nonfunctional by deletion of its RS motif and when this is replaced with the TRA RS motif this function is restored, indicating that this motif may play a similar role in both proteins (Li and Bingham, 1991). Deletion of the $Su(W^a)$ RS motif seemed to affect the nuclear localisation of the protein, which may suggest a possible role for this region. However, the $U2AF^{65}$ RS motif was also shown to be essential for its *in vitro* splicing activity (Zamore *et al.*, 1992), suggesting that this motif may have more than one function.

Regulation of tra-2 Splicing

The cloning of transformer-2 (*tra-2*) has revealed that it encodes four transcripts which are alternately spliced (Amrein *et al.*, 1988; Goralski *et al.*, 1989; Mattox *et al.*, 1990; Amrein *et al.*, 1990), as shown in Fig. 7. These transcripts potentially encode proteins with a common C terminus containing both the RS motif and the 80- to 90-amino-acid RNP motif, which is also found in SF2/ASF, SC35, SRp20, SRp75, and $U2AF^{65}$, as well as in the U1A and U2B″ snRNPs (reviewed in Kenan *et al.*, 1991), indicating that *tra-2* may also encode a splicing regulator.

tra-2 Function in the Male Germline

TRA-2 protein is required in the male germline, as shown by the fact that nonfunctional sperm are produced in flies mutant for this gene (Belote and Baker, 1983). Indeed, most abundant expression of *tra-2* is seen in this tissue where two male germline-specific transcripts are produced. In wild-type flies, the splicing of the M1 intron (shown in Fig. 7) is normally an inefficient process such that the concentration of the M1-containing transcript is higher than the


(a)
U2AF
?
AUG
UGA
UAA
1
2
3
4
5
6
7
8
9
10
Sxl Primary RNA
SXL
Sxl Processed RNA
UAG
tra Primary RNA
tra Processed RNA
TRA
TRA-2
dsx Primary RNA
dsx Processed RNA
N
C
COURTSHIP
REPRESSION OF MUSCLE
EXUPERANTIA
(b)
SPERMATOGENESIS

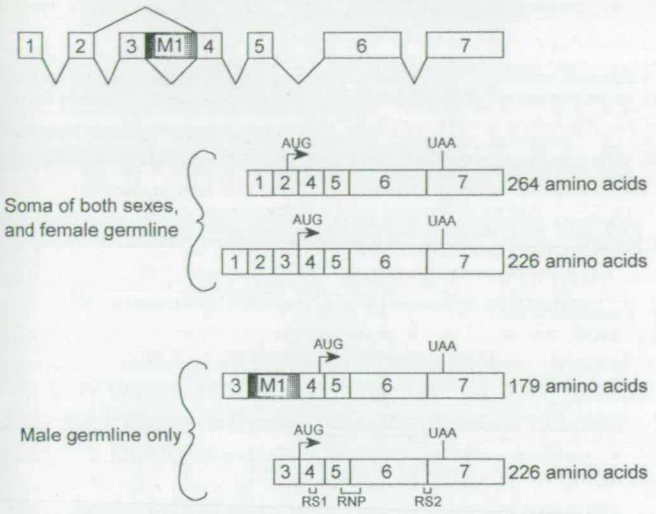


FIG. 7. Schematic diagram showing the alternate transcripts produced from the *transformer-2* locus. Exons are represented by open boxes. Removal of the M1 intron (shaded box) is thought to be inhibited by *tra-2* itself in the male germline. Tissue and sex specificity, as well as sizes of predicted proteins, are indicated for each transcript. RNP, ribonucleoprotein domain consensus sequence; RS1/RS2, arginine–serine-rich motifs.

completely spliced transcript. Analysis of the amounts of M1-containing transcript in *tra-2*$^-$ flies indicates that active *tra-2* product autoregulates this transcript, preventing the splicing out of the M1 intron which occurs to completion in the *tra-2* mutant flies (Mattox and Baker, 1991). This is supported by the observation that mutation of the M1 splice sites increases the levels of M1-containing transcripts. However, ectopic expression of M1-containing cDNA is not sufficient to produce mature sperm. Thus it may be that the repression of M1 splicing by active TRA-2 acts as a type of negative feedback mechanism to regulate the levels of TRA-2 in the male germline (Mattox and Baker, 1991).

tra-2 Regulates the Splicing of exu

It has been shown that one function of *tra-2* in the male germline is to regulate the production of male-specific transcripts from the gene *exuperantia* (*exu*). The *exu* gene has functions in both the male and the female germline (Hazelrigg *et al.*, 1990). In females, *exu* has a maternal effect, regulating the localisation of the *bicoid* gene product in the oocyte. In males, *exu* is required in the germline for correct spermatogenesis, since mutation of the gene results in male sterility. Two sex-specific and germline-dependent *exu* transcripts have been identified; a 2.9-kb transcript which is male germline-specific and a 2.1-kb transcript which is female germline-specific. These transcripts appear to be initiated from different promoters and exhibit sex-specific processing of the 3′ UTR region (Hazelrigg and Tu, 1994). In *tra-2* mutants' production of the *exu* male-specific transcript is much less efficient, although not totally abolished. Male-specific 3′ UTR sequences are important for spermatogenic *exu* function since deletions in this region result in male sterility.

Somatic Functions of tra-2

Although *tra-2* is clearly important for regulating male germline sexual differentiation, no somatic function has been demonstrated for *tra-2* in the male. In the female, however, *tra-2* acts in concert with *tra* to direct most aspects of female-specific differentiation (see Slee and Bownes, 1990, for review). Mutant alleles of *tra* or *tra-2* result in transformation of females into pseudomales exhibiting male characteristics. These include male pigmentation, cuticular structures, and rudimentary testes. They are, however, of female size and are infertile due to nonfunctional sperm. Mutations in the *tra* gene have no effect on males, and *tra-2* mutants show no male somatic transformations. Null alleles of the gene *dsx* result in intersexuality of both males and females, with the phenotype seeming to result from an expression of the genes responsible for both

FIG. 6. Schematic representation showing alternate splicing of *Sxl*, *tra*, and *dsx* transcripts. Exons are indicated by boxes, with open reading frames shaded. Proteins involved in splicing regulation and proteins produced from the processed transcripts are shown. Although only U2AF is shown as mediating normal splicing, in fact it is only one of a number of proteins that form part of the active spliceosome. (a) In females, SXL acts at poly(U) runs in the introns 5′ and 3′ of exon 3 to prevent inclusion of this exon in the processed mRNA. This results in production of a processed *Sxl* transcript capable of translating full-length SXL protein. SXL also blocks the acceptor site at the 5′ end of exon 2 in the *tra* unprocessed RNA. This results in an alternative downstream site being used instead. Thus, a *tra* processed mRNA is produced which can give rise to active TRA protein. TRA and TRA-2 proteins act together to stabilise the splicing apparatus at the acceptor site of exon 4 in the *dsx* unprocessed RNA. In this way, a female-specific *dsx*-processed RNA is produced which gives rise to female-specific DSX^F protein. Other functions of TRA and TRA-2 are also indicated. (b) Early SXL protein is absent in males, enabling inclusion of exon 3 in the *Sxl*-processed mRNA. This *Sxl* mRNA is incapable of producing full-length SXL protein. In the absence of SXL, the acceptor site at the 5′ end of exon 2 in the *tra* unprocessed RNA can be used. This yields a processed *tra* RNA which is incapable of producing active TRA protein. In the absence of both TRA and TRA-2 proteins together, the acceptor site at the 5′ end of *dsx* exon 4 is not used and a male-specific *dsx*-processed RNA is produced. This transcript gives rise to DSX^M male-specific protein. The functions of TRA-2 in male spermatogenesis are indicated. DSX^F, *doublesex* female-specific protein; DSX^M, *doublesex* male-specific protein; SXL, *sex-lethal* protein; TRA, *transformer* protein; TRA-2, *transformer-2* protein; U2AF, splicing factor U2AF.

male-specific and female-specific development at the individual cell level.

The epistatic relationship among *tra*, *tra-2*, and *dsx* was first shown by the construction of flies which carried double homozygous mutations in various combinations (Baker and Ridge, 1980). The epistatic gene could then be identified by virtue of its phenotype being manifest in the fly. This showed that *dsx* is epistatic to *tra*. In another set of experiments ectopic *tra* expression was shown to be unable to cause development along the female pathway in flies mutant for *tra-2* and *dsx*. In addition, molecular evidence shows that *tra* and *tra-2* are required for production of female-specific *dsx* transcripts (Nagoshi *et al.*, 1988). The above evidence shows that *dsx* is epistatic to *tra* and *tra-2* and that *tra* and *tra-2* are required for *dsx* to be expressed in the female mode, while the male functions of *dsx* are independent of *tra* and *tra-2*.

Regulation of dsx Splicing

The genetic evidence suggests that *dsx* is differentially active in both males and females, acting primarily to repress genes required for differentiation of the opposite sex. Analysis of the *dsx* transcripts showed how *tra* and *tra-2* enable this to occur (Baker and Wolfner, 1988; Burtis and Baker, 1989). Examination of cDNAs representing the 3.9-kb (male-specific) and 3.5-kb (female-specific) *dsx* transcripts shows that these messages are differentially spliced and polyadenylated but are both capable of producing large, functional proteins with sex-specific carboxyl termini. Hence it would be quite feasible for there to be differential activity in both sexes.

Germline transformants containing the female-specific *tra* cDNA fused to the hsp70 promoter have their male soma transformed to female soma. This transformation is correlated with the production of the female-specific *dsx* transcripts in the male soma, while tra^-, XX flies produce only male *dsx* mRNA (McKeown *et al.*, 1988). *tra-2* is also required for production of the female-specific *dsx* transcripts but not for production of male-specific *dsx* transcripts (Nagoshi *et al.*, 1988). Tissue culture cotransfection experiments (Hoshijima *et al.*, 1991; Ryner and Baker, 1991) allow the effects of TRA and TRA-2 proteins upon *dsx* pre-mRNA to be assessed directly by analysing the spliced products of *dsx* pre-mRNA in the presence or absence of TRA and TRA-2. These studies show that TRA and TRA-2 act in concert to positively promote the usage of the female-specific splice acceptor site, as shown in Fig. 6.

A region lying just downstream of the exon 4 acceptor site has been implicated as being involved in *tra* and *tra-2* regulation of the *dsx* pre-mRNA (Nagoshi and Baker, 1990). Lying in this region are six 13-nt repeats (*dsx* repeat element *dsx*RE), the deletion of which results in a loss of female-specific product in the cotransfection system described above (Hoshijima *et al.*, 1991; Ryner and Baker, 1991). It has been shown that TRA, TRA-2, and some SR proteins bind to the *dsx*RE *in vitro* (as shown in Fig. 6), with TRA-2 binding being dependent upon a purine rich enhancer (PRE) element present within the *dsx*RE (Hedley and Maniatis, 1991; Lynch and Maniatis, 1995). The same studies showed that the *dsx*RE and PRE elements act synergistically. Also, substitution of some of the noncanonical purines present in the polypyrimidine stretch of the female-specific acceptor site causes female-specific splicing independent of TRA and TRA-2 (Hoshijima *et al.*, 1991). This indicates that this splice site is not used in males because of its nonstandard polypyrimidine stretch. In females, TRA and TRA-2 act to stabilise the splicing apparatus at this site and thus promote its use, i.e., default splicing occurs in males, while in females regulation of female-specific splicing occurs by TRA and TRA-2 promoting the use of the nonpreferred site. Indeed, it has been demonstrated that TRA and TRA-2 work by attracting general splicing factors, including some SR proteins, to the *dsx*RE region, enabling it to function as a splicing enhancer (Tian and Maniatis, 1993).

Sequences homologous to the *dsx*RE 13-nt repeats have been identified in both *exu* and *tra-2* transcripts. Since, in the male germline, alternative processing of both these transcripts is under the control of *tra-2*, it would be expected that mutations of the repeat sequences would result in loss of *tra-2* regulation. However, mutational analysis of some of these sites has not as yet been able to establish a role for them in sex-specific splicing regulation (W. Mattox and T. Hazelrigg, personal communication).

The RNP motif of TRA-2 has been shown to be essential for its somatic and male germline functions, although it is required but not sufficient to direct RNA binding *in vitro* (Amrein *et al.*, 1994). Experiments using the yeast dihybrid assay have shown that TRA and TRA-2 physically interact with themselves, with each other, and with the general splicing factor SF2. These three proteins have been shown to be sufficient to cause *dsx* primary transcripts to be spliced in a female-specific manner. One of the TRA-2 RS motifs, RS2, is essential for *in vivo* function and for interactions in the dihybrid assay. The RS1 RS motif of TRA-2 is not essential but, if deleted, results in temperature-sensitive mutation *in vivo* and decreased sensitivity of dihybrid interactions *in vitro* and so may act as a stabiliser of protein–protein interaction (Amrein *et al.*, 1994).

In addition to promoting the usage of certain splice sites, TRA-2 also appears to be able to prevent the usage of splice sites. This is shown by the fact that *tra-2* downregulates the removal of the M1 intron from *tra-2* primary transcripts in the male germline (Mattox and Baker, 1991). This may be a result of the proximity of the splice site relative to the TRA-2 binding site, which may cause bound TRA-2 to interfere stearically with the splicing apparatus. This repressive function may alternatively be due to additional tissue-specific factors which modulate TRA-2 function.

Additional Functions of tra

For most aspects of somatic sexual differentiation, *dsx* is the last member in the hierarchy of regulatory genes. This

view, however, in which the only sex determination function of *tra* and *tra-2* is to direct the *dsx* primary transcript to be spliced in the female mode, needs to be revised. Fresh evidence indicates that *tra* and *tra-2* also govern *dsx*-independent pathways of sex-specific differentiation.

Male flies have a sex-specific pair of muscles known as the muscle of Lawrence (MOL) (Lawrence and Johnston, 1984). These muscles span the fifth abdominal segment and were initially thought to be involved in the curling of the male abdomen during copulation. More recently, however, it has been found that flies lacking the MOL can still copulate (Gailey *et al.*, 1991). XX flies carrying null mutations of *tra* or *tra-2* develop as pseudomales which have this muscle present (Taylor, 1992). However, this effect of the *tra* and *tra-2* alleles cannot be due to their function in *dsx* regulation, as the muscle is present in XY individuals mutant for *dsx* but is absent in XX *dsx* mutant flies. Thus, the repressive function that active TRA and TRA-2 proteins exert on the development of this muscle must act via a pathway that is independent of *dsx*. Transplantation of nuclei between males and females has shown that the identity of this muscle is not autonomous but depends upon the sex of the innervating axons (Lawrence and Johnston, 1986).

Recent studies on courtship behaviour have also pointed towards the presence of at least one branch of regulatory genes which are governed by *tra* and *tra-2* but not by *dsx*. In wild-type flies, mating involves a number of male-specific courtship behaviours which are readily observable (Spieth, 1974; Ehrman, 1978). Courtship is initiated with the male tapping the female with his forelegs, orienting toward her, and following her. He then begins a courtship song by extending one wing and vibrating it. This is followed by the male extending his proboscis and licking the female's genitalia and finally copulation is attempted. Females are largely sedentary during mating, although reception and rejection behaviours are observed.

Temperature shift experiments using a temperature-sensitive allele of *tra-2* have shown that absence of male behaviour in the female is dependent upon activity of the *tra-2* gene, such that inactivation of this gene in females from the late larval stage onwards results in appearance of male courtship elements (Belote and Baker, 1987). Flies of the genotype XX;dsx^- exhibit no male courtship behaviour suggesting that DSX^F does not normally act to repress male courtship behaviour in the female. Similarly XX;dsx^D flies which constitutively express DSX^M do not attempt to court despite the fact that they are male in morphology. This indicates that DSX^M does not activate male courtship in the male (Taylor *et al.*, 1994). These experiments argue that there is no role for *dsx* in regulating courtship behaviour. However, it has been observed that XY;dsx^- flies court much less than normal males. These flies also elicit more courtship than wild-type adult males, even though such flies do not produce characteristically female pheromones (McRobert and Tompkins, 1985; Jallon *et al.*, 1988). At first glance, this would indicate a direct role for *dsx* in regulating this behaviour. Taylor *et al.* (1994) observed, however, that although a large proportion of XY;dsx^- flies did not court at all, at least one fly carrying each tested allele exhibited male courtship behaviours up to and including wing extension. The lack of attempted copulation of XY;dsx^- flies may be explained by their morphology, which makes this a physical impossibility. This led Taylor *et al.* (1994) to propose that the anomalous expression of both male- and female-specific genes in dsx^- flies, due to lack of the repressive function of both DSX^M and DSX^F proteins, may lead to developmental abnormalities, resulting in the XY;dsx^- fly being less able to sense attractive females. Thus, even though the neural identity of the fly remains male, it would be less likely to court. This may also account for the increased sex appeal of these flies. Young wild-type males show both a lower courtship frequency and a higher elicitation of courtship than do wild-type adult males. The developmental burden caused by expression of both male- and female-specific genes in the XY;dsx^- flies may result in a retardation of maturation which causes these flies to retain their sex appeal and low courtship frequency after the time when wild-type males would have lost theirs. Evidence that the CNS of XY;dsx^- flies is essentially male comes from analysis of the courtship song which, although not exactly wild type, is still clearly recognisable. This is in marked contrast to the anomalous song produced by gynandromorphs when much of the thoracic nervous system is diplo-X (Taylor *et al.*, 1994).

Taken together, the above evidence suggests that *tra* and *tra-2* regulate the sexual identity of the fly regarding courtship behaviour, but *dsx* does not appear to be required, implying a branch of regulatory genes under the control of *tra* and *tra-2* but not *dsx*.

A candidate gene for being involved in such a pathway is the *fruitless* (*fru*) gene, which has marked effects on male courtship behaviour (Gailey *et al.*, 1991; Taylor *et al.*, 1994). Males with extreme *fru* alleles court vigorously but do not attempt copulation and are unable to curl their abdomen. Defects are also observed in the courtship song of these mutants. With regard to the possibility that this gene may be involved in a pathway of regulation governed by *tra* and *tra-2* but not *dsx*, two aspects of the *fru* phenotype are particularly interesting. First, certain alleles result in the absence of the MOL, although this does not account for the inability of these flies to curl their abdomen since weaker *fru* alleles which lack this muscle are still able to copulate. As discussed above, *tra* and *tra-2* are required to prevent the formation of this muscle in the female, while *dsx* and *ix* are not. However, whether *tra* and *tra-2* play any part in *fru* regulation is unknown. Second, severe *fru* alleles result in male flies courting nonspecifically such that males are courted with equal vigour as females. Interestingly, lack of courtship discrimination by male flies has also resulted from ectopically expressing *tra* in the antennal lobes or mushroom bodies (Ferveur *et al.*, 1995; O'Dell *et al.*, 1995). Again, although mutation of *fru* and missexpression of *tra* have similar effects with regard to loss of courtship discrim-

ination in the male, it is not known whether these two genes form part of a common pathway.

The localised expression of *tra* described above was achieved using a *tra* cDNA under the control of the yeast Upstream Activator Sequence (UAS). This construct was introduced into the genome via P-element-mediated germline transformation. To express *tra* in a specific tissue, it is necessary to produce the yeast UAS-activator protein GAL4 in that tissue. This was done by transforming embryos with a P-element construct containing a GAL4 cDNA under the control of a weak promoter. This weak promoter requires the "help" of a tissue-specific enhancer to express significant levels of GAL4. Thus, the tissue localisation of GAL4 protein in these "enhancer-trap" strains depends entirely upon which tissue-specific enhancers the P-element construct comes under the control. By selecting strains which express GAL4 brain specifically and crossing them to the UAS–*tra* strain, brain-specific expression of *tra* can be achieved.

It is becoming increasingly clear that what was previously thought to be a linear hierarchy of regulatory genes is in fact a branched pathway, with sex differentiation genes lying directly under the control of *tra* and *tra-2* as well as *dsx*. It is likely that there are a number of different branches at the level of *tra* and *tra-2* and the elucidation of these processes will no doubt be the basis of future work.

The Control of Male and Female Sexual Differentiation by dsx

The major morphological differences between males and females are apparent during differentiation of the adult at metamorphosis. Many genes expressed uniquely in adult male or female somatic cells have been identified. These include the components of the vitelline membrane and chorion in females (e.g., Waring and Mahowald, 1979; Fargnoli and Waring, 1982; Kafatos *et al.*, 1985) and components of the accessory gland in males (e.g., Schäfer, 1986; Chen *et al.*, 1988; Monsma and Wolfner, 1988; DeBenedetto *et al.*, 1990). Yet most of the genes encoding these sex-specific proteins are not directly controlled by the *dsx* gene in the adult. All of the products mentioned above are made in sexually unique tissues or organs and the regulation of the genes encoding them depends upon the presence or absence of tissue-specific factors. The *dsx* gene has played its role earlier in development by determining and maintaining the state of determination throughout embryonic and larval growth and is no longer required once the cells differentiate. The exceptions to this are the *yolk protein* (*yp*) genes expressed in the female (but not the male) adult fat body (Bownes and Nöthiger, 1981) and the *glucose dehydrogenase* gene expressed in a specific pattern in the male and female reproductive tract (Feng *et al.*, 1991). Both these examples are of genes expressed in a tissue found in adults of both sexes, but with some unique sexually dimorphic functions, showing that *dsx* can function after differentiation to control sex-specific gene expression in certain tissues.

The major developmental decisions executed by *dsx* therefore occur during embryonic and larval development. Its functions are well documented by genetic and developmental studies, such as the analysis of mosaics and gynandromorph flies which contain cells of each sex within the same organism (Schüpbach *et al.*, 1978). The role of *dsx* is perhaps best illustrated for the genital disc, which gives rise to the adult analia and genitalia and comprises three distinct groups of primordial cells. One group will eventually differentiate into either male or female analia depending upon whether DSX^F or DSX^M is expressed. There is, however, a selection between the other two groups of cells, with one group growing in the female and the other in the male and differentiating into the very different genitalia of the two sexes. This means that *dsx* is able to select either the repression of or promote the growth of whole primordia as well as cause a single group of cells to select between two alternate developmental pathways (Epper and Nöthiger, 1982). How this is achieved is not clear but presumably depends upon the position of the primordial cells in the fly and upon interaction with the segment polarity and/or the segment identifying homeotic genes. Figure 8 shows the development of the male and female genital primordia, discs, and structures generated at metamorphosis in males and females.

Analysis of mosaic patches of sexually transformed cells and temperature shift experiments with a temperature-sensitive allele of *transformer-2* (*tra-2*^{ts}) shows that *dsx* is required throughout the growth of genital discs, functioning not only to set the cells along a specific developmental pathway, but also to maintain that determined state throughout the subsequent cell divisions. Thus *dsx* is required to maintain the appropriate sexual determination of cells (Wieschaus and Nöthiger, 1982; Epper and Bryant, 1983). Consistent with these results is the observation that *dsx* mutant flies differentiate both male and female pattern elements, indicating that both groups of primordial cells develop to some extent when neither DSX^F or DSX^M protein is present (Nöthiger *et al.*, 1987).

The main activity of DSX^F and DSX^M seems, from genetic studies, to be to repress the expression of genes needed in the other sex. There are, however, some indications that the proteins may also promote sex-specific gene expression. There are sex-specific differences in the cell divisions which generate the abdominal neurons of the adult. Once the female neuroblasts stop dividing, the terminal abdominal neuroblasts of the male undergo extra divisions. In the absence of *dsx* (in contrast to the genitalia) the cells do not divide at all in either sex. Thus *dsx* is required for the non-sex-specific cell divisions prior to the sexually dimorphic cell divisions (Taylor and Truman, 1992).

The male foreleg carries the sex comb and the pattern of neuronal axons differs between the male and female first legs. Recent experiments using ectopic expression of a *dsx* male-specific cDNA in flies showed that sex comb morphol-

ogy could be induced not only in female forelegs but also on second and third legs of both sexes. This has been used to indicate that DSX^M positively promotes the development of male-specific structures of the foreleg (Jursnich and Burtis, 1993). However, this should be viewed with care, since *dsx* nulls do not have a similar phenotype and the dsx^D mutants, which constitutively express the male DSX protein, do not have sex combs on other legs. It is possible that *dsx* is not normally expressed at all in these cells and high levels of expression may lead to new phenotypes by interactions with new combinations of tissue-specific transcription factors.

One interesting point to note is that a lack of DSX proteins, or an expression of both forms of DSX^F and DSX^M in the same fly, leads to a similar phenotype, namely intersexual flies. When no DSX protein is present, presumably the sex-differentiation genes of both sexes are derepressed and thus both sets of genes are expressed. When both gene products are present it is possible that they interfere with each other's function, again leading to a partial derepression of the sex-differentiation genes.

Although *dsx* is not involved in sex determination within germ cells, its correct expression is essential in somatic cells of the gonad for germ cells to be able to develop along a female developmental pathway (Steinmann-Zwicky, 1992). Thus there is signalling between somatic cells and germ cells during development of the germline and gonad to ensure compatibility between germ cell and somatic cell sex differentiation (see section on *Sex-lethal* and germline sex determination).

Whilst we know a great deal at the level of genetic decisions about developmental fate control by the alternately spliced products of the *dsx* gene, we have no idea what the target genes of DSX^F and DSX^M are within imaginal primordia and histoblasts during development. In fact the only immediately identified downstream targets of *dsx* are the female-specific *yolk protein* genes expressed in the adult fat body. The *glucose dehydrogenase* gene expressed in the reproductive tract may also be a target but this has not been investigated at the molecular level.

For normal female differentiation to occur, two other genes are required. These are *ix* (Baker and Belote, 1983) and *her*, both of which have zygotic functions essential for normal female differentiation, yet act downstream and independently of the normal sex-specific splicing of *dsx* (Pultz *et al.*, 1994; Pultz and Baker, 1995). It seems likely that these gene products interact with DSX^F to bring about female-specific differentiation. The molecular nature of *ix* remains to be elucidated, but it is clear that *dsx* alone does not generate the developmental decisions during embryonic and larval development.

Execution of the Signal: Interactions of dsx with Downstream Sex Differentiation Genes

Despite the wealth of information about the developmental consequences of mutations in *dsx* we have information only about its molecular interactions with one family of downstream genes, encoding the female-specific yolk proteins. Even with these genes we really don't understand the nature of sexual selection, since DSX^F and DSX^M bind to the same DNA fragment and give the same footprint, showing that they contact the same DNA bases (Burtis *et al.*, 1991), yet *in vivo* this leads to *yolk protein* gene repression in males and expression in females.

The three *yolk protein* genes (*yp1*, *yp2*, and *yp3*) are expressed in the follicle cells of the ovary and in the adult female fat body (reviewed in Bownes, 1994). Their expression in the follicle cells is not directly controlled by *dsx* but is cell-type-specific once the *dsx*-dependent decision to develop and differentiate an ovary rather than a testis has been made (Bownes *et al.*, 1990). In the fat body, *yp* gene expression is directly controlled by the sex-determination hierarchy (Belote *et al.*, 1985). XX flies with mutations in *tra-2* or *dsx*, for example, can have their *yp* genes repressed and XY flies with mutations at *dsx* can express the *yp* genes in the fat body. This latter finding suggests that the main function of DSX^M is to repress expression in males. There is some evidence that DSX^F promotes *yp* gene expression in females (Coschigano and Wensink, 1993).

A number of *cis*-acting DNA sequences have been identified which confer female fat body expression of *yp1*, *yp2*, and *yp3*, using a variety of reporter gene systems (Garabedian *et al.*, 1985, 1986; Logan *et al.*, 1989; Logan and Wensink, 1990; Liddell and Bownes, 1991; Abrahamsen *et al.*, 1993; Ronaldson and Bownes, 1995). One of these, a 125-bp fragment located 5′ of *yp1*, called the fat body enhancer (FBE), has been extensively studied, but it should be noted that it is not the only region flanking *yp1* and *yp2* that can direct female fat body expression of these two divergently transcribed genes. DSX protein, which contains a zinc finger DNA binding domain, binds four times within the FBE. DSX^F and DSX^M differ at their carboxy termini, not in the DNA binding region, and can compete for the same DNA binding sites in the FBE (Burtis *et al.*, 1991; Erdman and Burtis, 1993).

A number of other proteins, fat body transacting factors, have been shown *in vitro* to bind to the FBE (Abel *et al.*, 1992: Falb and Maniatis, 1992). These include both enhancers and repressors, the transcriptional activator box B-binding factor-2, the CCAAT/enhancer binding protein, and the adult enhancer factor 1. These proteins regulate *alcohol dehydrogenase* gene expression in the fat body and give footprints which overlap with those of DSX in the FBE.

It seems that the interaction with these other trans-acting factors may differ according to whether DSX^F or DSX^M is present. When neither is present transcription will proceed. When DSX^F alone is present, transcription also proceeds, but when DSX^M alone binds, transcription is repressed. Perhaps DSX^M is less easily displaced by transcriptional activators in the fat body. The presence of both DSX^F and DSX^M allows transcription of the *yp* genes, possibly because they interfere with each other's function.

To understand how this is achieved will require a combi-

nation of *in vitro* gel shift and footprint assays to investigate which combinations of factors will bind or be displaced by each other. Mutations induced in the binding sequences will then be essential to see if these *in vitro* studies are valid by analysis of the *in vivo* function of the altered binding sites. Other, as yet unidentified, gene products, such as those encoded by *ix* (Baker and Belote, 1983), may also have critical roles to play in the sex specificity of *yp* gene expression. Figure 9 shows our current understanding of the role of *dsx* in *yp* gene expression.

Ovarian development requires interaction between the germ cells and somatic cells. We know that signals sent from the sexually determined female somatic cells to the germ cells are essential for female germline development. A gene which is downstream of *dsx* in the sex determination pathway and which is essential for ovarian and female germline development is *small ovaries (Wayne et al.*, 1995). This mutation maps to the X-chromosome and once cloned would be a good possibility for an additional target for *dsx* gene regulation.

CONCLUSIONS

Alternate splicing provides a mechanism for generating families of related proteins with either dramatic or subtly different functions, depending upon the system. In *Drosophila* this has been exploited to regulate sexual determination and differentiation by a genetic cascade of alternately spliced products. The alternate splicing of the transcripts encoding both splicing factors and DNA binding proteins is quite well understood, but there are a number of branches in the pathway that remain to be elucidated, along with understanding the mechanism by which the differentially spliced products of the DNA binding proteins can bring about sex-specific transcription of target genes.

ACKNOWLEDGMENTS

We are grateful to Isobel Black and Josie Priestley for typing and manuscript preparation; to Daniel Bopp, Jean Beggs, Rolf Nöthiger and Neil White for providing valuable comments on the manuscript; and to Daniel Bopp, T. Hazelrigg, and W. Mattox for providing photographs and sharing unpublished data. Research in our laboratory is supported by the MRC and bbsrc.

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Received for publication September 6, 1995